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Molecular Approaches to the Study of Marine Cyanophages

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Declaration

I hereby declare that the work described in this thesis was conducted by myself, under the supervision of Dr. N. H. Mann and Dr. I. R. Joint, with the exception of those instances where the contribution of others has been specifically acknowledged.

None of the information contained herein has been used in any previous application for a degree.

All sources of information have been specifically acknowledged by means of reference.

Nicholas Jonathon Fuller

Summary

Cyanophages are thought to play an important role in the mortality and clonal composition of marine *Synechococcus* spp., and have been shown to be widespread throughout the world's oceans. However, relatively little research has been made into the molecular analysis of marine cyanophages. This study continued previous research to develop molecular probes (PCR primers) which would specifically detect cyanophages which infect marine *Synechococcus* spp., and be used to interrogate natural marine cyanophage populations. An attempt was made to develop a rapid technique for quantifying marine cyanophages, using competitive PCR (cPCR).

For the development of cyanophage-specific PCR primers, several cyanophages which infected *Synechococcus* sp. strains WH7803 and WH8018 were isolated from coastal Bermuda and the Sargasso Sea. A region of DNA had previously been found which showed homology amongst several marine cyanophages, and to T4 gene 20, which encodes a minor capsid protein. Homologues from three cyanophages were completely sequenced, and two, potentially cyanophage-specific, PCR primers were designed.

The primers detected only marine cyanophages which belonged to the family Myoviridae, regardless of the geographical location of their isolation. They also detected cyanophages which infected different marine *Synechococcus* spp. strains, and therefore provide a more comprehensive tool than infective methods. The primers were able to detect as few as 190 cyanophages μl^{-1} , which would correspond to an *in situ* concentration of 10^3 PFU ml^{-1} . The PCR should therefore detect most natural concentrations of marine cyanophages in surface waters, especially with prior concentration from seawater. Preliminary experiments showed that PCR products could be obtained from as little as 1 μl of unconcentrated seawater. PCR therefore provides a sensitive method for the detection of marine cyanophages, which is far more rapid than traditional infection techniques.

Quantification by cPCR was attempted. An internal competitor was constructed, and a calibration curve was drawn for three cyanophages, with a log-linear relationship over *ca.* three orders of magnitude of cyanophage numbers. This demonstrates that rapid quantification of a known marine cyanophage is possible. However, cPCR of the three different cyanophages resulted in three different calibration curves. Hence, quantification of a marine sample containing a mixture of cyanophages was not yet possible.

The cyanophage-specific primers were then applied to marine samples which were collected whilst on the AMT-2 cruise, from Port Stanley (Falkland Islands) to Plymouth (UK). Cyanophages were concentrated by tangential flow filtration, and PCR products were obtained from most of the surface samples throughout the Atlantic Ocean. Products from some of the stations were sequenced, providing novel genetic information of natural marine cyanophage populations.

The results showed that cyanophage populations were highly diverse, with at least twelve genetically different cyanomyoviruses in one sample. Some sequences obtained from the same sample were clearly very similar to each other, whilst others within a sample could be as diverse as those isolated from different oceans. However, very similar sequences were obtained from some samples separated by thousands of miles, in different hemispheres, or even in different oceans.

Abbreviations

A	absorbance	HPLC	high performance liquid chromatography
AMPS	ammonium persulphate		
AMT	Atlantic meridional transect	HSV	herpes simplex virus
APC	allophycocyanin	IPTG	isopropyl- β -d-thiogalactopyranoside
ASW	artificial seawater		
ATP	adenosine triphosphate	kb	kilobase pairs
bp	base pairs	kDa	kilo Daltons
BBSR	Bermuda Biological Station for Research	MC	marine cluster
BSA	bovine serum albumin	MOI	multiplicity of infection
<i>ca.</i>	<i>circa</i>	NW	north-west
CFU	colony-forming units	PAGE	polyacrylamide gel electrophoresis
CIP	calf intestinal phosphatase	PAR	photosynthetically active radiation
cPCR	competitive PCR		
CPS	cyanophage-specific	PBS	phosphate-buffered saline
CTD	conductivity, temperature, depth	PC	phycocyanin
Da	Daltons	PCC	Paris culture collection
DAPI	4, 6-diamidino-2-phenylindole	PCR	polymerase chain reaction
dATP	deoxyadenosine triphosphate	PEG	polyethylene glycol
dCTP	deoxycytidine triphosphate	PFU	plaque-forming units
D-DNA	dissolved DNA	PHYLP	phylogeny inference package
DGGE	denaturing gradient gel electrophoresis	POC	particulate organic carbon
dGTP	deoxyguanosine triphosphate	PVP	polyvinylpyrrolidone
DMF	N,N-dimethylformamide	RFLP	restriction fragment length polymorphism
DMS	dimethylsulphide	RNA	ribonucleic acid
DMSP	dimethylsulphonopropionate	rRNA	ribosomal RNA
DNA	deoxyribonucleic acid	RRS	royal research ship
dNTP	deoxyribonucleotide triphosphate	RSV	respiratory syncytial virus
DOC	dissolved organic carbon	SDS	sodium dodecyl sulphate
DOM	dissolved organic matter	SE	south-east
DOP	dissolved organic phosphate	sp.	species (singular)
DSC	discrimination of secondary structure class	spp.	species (plural)
DTT	dithiothreitol	SSC	sodium chloride-sodium citrate
dTTP	deoxythymidine triphosphate	SSPE	sodium chloride-sodium dihydrogen phosphate
EDTA	ethylenediaminetetra-acetic acid	SW	south-west
ESW	enriched seawater	TBE	Tris-borate-EDTA
G+C	guanine + cytosine	TEM	transmission electron microscopy
GCG	Genetics Computer Group	TEMED	N,N,N',N'-tetramethylethylenediamine
gp	gene product	Tris	Tris (hydroxymethyl) methylamine
GTE	glucose-tris-EDTA	TYE	tryptone yeast extract
HAV	hepatitis A virus	UV	ultraviolet
HA-Y	Hans Analytik-Yellow	VBR	virus to bacteria ratio
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]	WH	Woods Hole Oceanographic Institution culture collection
HIV	human immunodeficiency virus	XGal	5-bromo-4-chloro-3-indolyl- β -galactoside
HNF	heterotrophic nanoflagellate		

Chapter 1:

Introduction

1.1 The Marine Environment

The marine environment is increasingly being studied, as its importance to economy and global ecology is being revealed. The oceans cover about 70% of the surface of this planet, with a mean depth of 3.7 km, although they are between 4 and 5 km deep for much of this coverage. However, despite this potentially vast capacity for biological systems, relatively little is known about the biology of the oceans.

Sunlight is filtered out, absorbed by the water and any particles or organisms present, largely in the surface 100 m (the depth depending on water clarity) (see review by Kirk, 1992). As depth increases, the spectral composition of the light changes, with the water quickly absorbing red, green and UV light. Blue light penetrates the deepest, and is reflected back up, giving the oceans their deep blue colour. Below *ca.* 100 m there is insufficient light for photosynthetic organisms to reproduce (*e.g.* see review by Waterbury *et al.*, 1986). Hence, most of the biological activity of the oceans occurs in the surface 100 m, termed the euphotic zone, and the vast majority of the water column is dark, cold, under high pressure and contains comparatively little life. Much of this biological activity is in the nutrient-rich coastal waters, whilst much of the expanse of the open oceans is oligotrophic (nutrient-poor) and much more sparsely populated, even in the surface euphotic zone (see review by Lalli and Parsons, 1993).

Physical stratification of the water column occurs when the sun warms the surface waters, making them less dense. A stable thermocline results, with a body of warmer, well-mixed water floating above, and separated from, the colder, less well-mixed water below. The surface-mixed layer above the thermocline may be between 1 and 100 m deep, and provides a physically distinct habitat for biological activity, which generally decreases considerably below the thermocline (for review see Lalli and Parsons, 1993). Stratification, followed by mixing, occurs in seasonal and diel cycles, resulting from changes in illumination, convection and wind-driven turbulence. The long-term stability of a thermocline often results in the nutrients being depleted in the

surface-mixed layer as a result of biological activity; whereas the water below the thermocline is nutrient-replete. Hence, mixing of the two water bodies, when the thermocline breaks down, results in fresh nutrients being brought to the surface waters (see review by Lalli and Parsons, 1993).

Many micro-organisms can play vital roles in the climate and ecology of this planet (see review by Holligan, 1992). Indeed, total primary production from the oceans accounts for a large amount of total global primary production. For example, it is thought that microbial production of dimethylsulphide (DMS) in the oceans has a key role to play in a negative feedback system to maintain a relatively stable global temperature, via the oxidation of DMS into various acidic compounds in the atmosphere. These acids form cloud condensation nuclei, resulting in the reflection of the sun's radiation away from the earth (see review by Malin *et al.*, 1992).

Biogeochemical cycles, such as those for nitrogen and phosphorus, have globally significant components in the marine environment (*e.g.* see review by Follmi, 1996). Bacteria are essential in the oxidation and reduction of nitrogen-containing compounds in the oceans. Bacteria and phytoplankton are involved in converting inorganic nitrogen to organic nitrogen (see review by Caron, 1994), whilst dissolved nitrogen gas is fixed to organic compounds notably by cyanobacteria in the surface waters (*e.g.* see review by Capone *et al.*, 1997). Primary production in the euphotic zone results partly from recycled nitrogen in the euphotic zone. However, "new production" (Dugdale and Goering, 1967) also occurs, resulting from nitrate outside the euphotic zone entering this zone, mainly from below by vertical mixing. For a review of the marine nitrogen cycle see Lalli and Parsons (1993). Since phosphorus is an essential nutrient, in the form of phosphate, its cycle has close links to that of nitrogen. For a review of the phosphorus cycle see Follmi (1996).

1.2 Marine Cyanophages

Cyanophages, viruses which infect cyanobacteria, have been known for a long time in freshwater (Safferman and Morris, 1963), and have been extensively characterised (for review see Cannon, 1987). However, very few cyanophages of freshwater unicellular cyanobacteria have been isolated (Safferman *et al.*, 1983; Kim and Choi, 1994), suggesting there are low cyanophage concentrations in this environment. Particular interest was placed in them because of the possibility of controlling nuisance cyanobacterial blooms (see review by Cannon, 1987). The importance of cyanobacteria as contributors to oceanic primary production has been well documented (Waterbury *et al.*, 1986). It is therefore surprising that relatively little research has been made into the occurrence and importance of marine cyanophages.

1.2.1 Abundance, distribution and diversity

Proctor and Fuhrman (1990) first reported counts of marine cyanobacteria in the final stage of lytic infection by cyanophages, and observed that up to 5% of cyanobacteria in different marine locations contained mature cyanophages, as visible by TEM. Since then most of the research into marine cyanophages has concentrated upon cyanophages which infect marine *Synechococcus* spp., although cyanophages have also been isolated which infect *Phormidium persicinum* (Ohki and Fujita, 1995; 1996) and *Trichodesmium* sp. (Ohki, 1997). It has become apparent that *Synechococcus* spp. cyanophages are ubiquitous in the world's oceans, like their host. They have been isolated from the Gulf of Mexico, the north-west Atlantic, the Sargasso Sea, the English Channel, the North Sea and the Red Sea (Suttle and Chan, 1993; Waterbury and Valois, 1993; Wilson *et al.*, 1993; Suttle and Chan, 1994; Wilson, 1994).

Marine cyanophages infecting *Synechococcus* spp. have often been observed at concentrations $>10^5$ ml⁻¹ (Suttle and Chan, 1993; Suttle *et al.*, 1993; Wilson *et al.*,

1993; Suttle and Chan, 1994). Cyanophage abundance appears to follow that of its host (Waterbury and Valois, 1993), and is highest near the surface and in coastal waters, where *Synechococcus* spp. concentrations correlate with light and nutrient requirements (Suttle and Chan, 1993; Suttle *et al.*, 1993; Suttle and Chan, 1994). Cyanophage concentrations were observed to decrease below the thermocline (Suttle and Chan, 1994), but have been observed in oceanic regions at 97 m depth at 10^2 ml⁻¹ (Suttle and Chan, 1994). On a transect in the Gulf of Mexico Suttle and Chan (1993) observed that cyanophage concentrations near the shore were *ca.* 10^4 ml⁻¹, decreasing to *ca.* 10^3 ml⁻¹ further offshore. Cyanophages have been detected over a wide range of water temperatures (12-30.4°C) and salinities (18-70 ‰), but are generally most abundant at the highest temperatures and lower salinities found in coastal regions, resulting from the distribution of their host (Suttle and Chan, 1993; 1994).

Suttle and Chan (1994) observed that cyanophage concentrations were greater than those of their host, even by up to 6-8 times. They also observed a threshold in *Synechococcus* spp. concentrations of *ca.* 10^3 ml⁻¹, above which there was a considerable increase in cyanophage concentrations (10^3 -fold) with just a slight increase in host concentrations (Suttle and Chan, 1994). In a seasonal study of cyanophages infecting two *Synechococcus* sp. strains in Woods Hole harbour, north-west Atlantic, Waterbury and Valois (1993) observed that cyanophage concentrations increased from winter low values of undetectable or <100 ml⁻¹, to summer values of 10^3 - 10^4 ml⁻¹ (Fig. 1.1). The increase in cyanophage abundance began about a month after the onset of the *Synechococcus* spp. spring bloom (Waterbury and Valois, 1993), corresponding to host concentrations having exceeded the required threshold for efficient cyanophage multiplication (Suttle and Chan, 1994). In contrast to the observations of Suttle and Chan (1994), cyanophage concentrations remained about an order of magnitude less than their host (Waterbury and Valois, 1993).

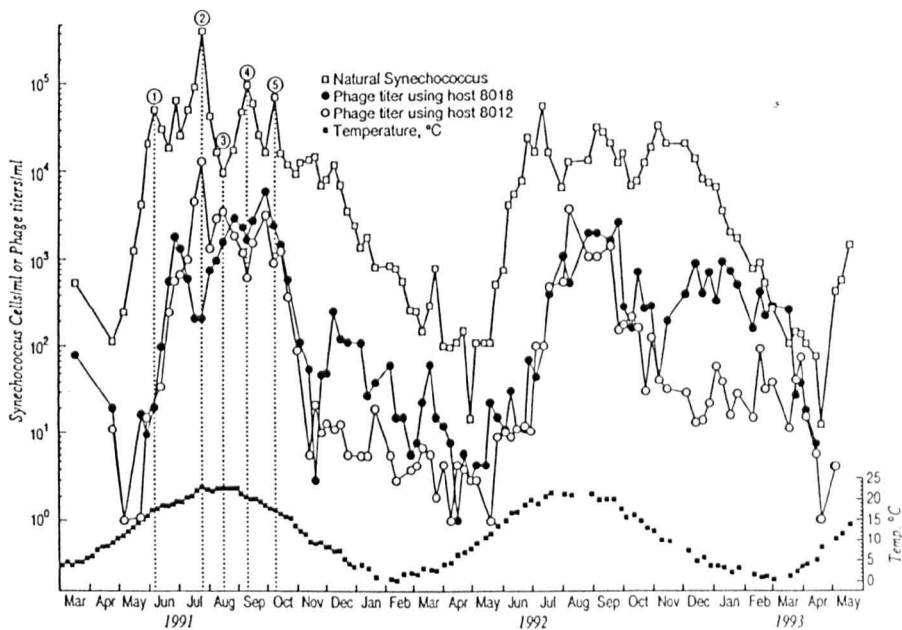


Figure 1.1 Annual cycle of *Synechococcus* spp. and *Synechococcus* spp. cyanophages in Woods Hole Harbour. Cell counts were determined by epifluorescence microscopy. Cyanophage titres were determined by the most-probable-number technique in tissue culture wells with the host strains *Synechococcus* spp. WH8018 and WH8012. These strains were chosen because of their sensitivity to a broad spectrum of *Synechococcus* spp. cyanophages (reproduced from Waterbury and Valois, 1993).

Cyanophage abundances are determined by infective techniques and counts are greatly affected by the host strain used. Suttle and Chan (1993; 1994) generally found that cyanophages which infect phycoerythrin-containing *Synechococcus* sp. strains, in particular *Synechococcus* sp. strains WH7803 (DC2) and WH6501 (SYN48), were considerably more abundant than cyanophages which infect other *Synechococcus* sp. strains. The results of Suttle and Chan (1993; 1994) suggest that oceanic *Synechococcus* sp. strains are more susceptible to infection than coastal strains. Waterbury and Valois (1993) observed that the majority of *Synechococcus* spp. clones in natural communities were resistant to the majority of their co-occurring cyanophages. In the light of this observation Suttle and Chan (1994) suggested that the greater concentrations of hosts and cyanophages in coastal waters than offshore would result in more frequent contact and therefore greater selection

for resistance to cyanophages in coastal strains. Hence, this would explain the greater susceptibility of oceanic strains to cyanophages.

Freshwater cyanophages were identified as belonging to the bacteriophage families Myoviridae, Siphoviridae and Podoviridae, and were classified as distinct genera by Safferman *et al.* (1983); now known as *Cyanomyoviridae*, *Cyanosiphoviridae* and *Cyanopodoviridae*. A schematic representation of the morphological structure of cyanophages typical of these genera is shown in Figure 1.2.

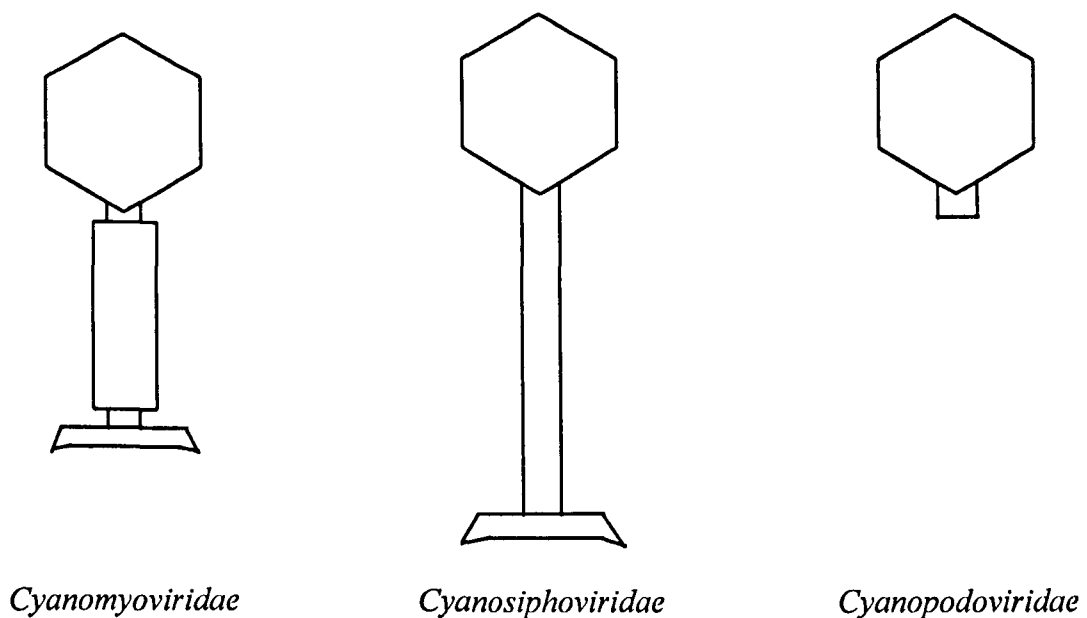


Figure 1.2 Schematic representation of typical members of the three cyanophage genera. *Cyanomyoviridae* isolates have a contractile tail; *Cyanosiphoviridae* isolates have a long non-contractile tail; and *Cyanopodoviridae* isolates have a short non-contractile tail.

Marine cyanophages have been observed to be a very diverse group of viruses (Waterbury and Valois, 1993). Waterbury and Valois (1993) isolated and characterised over fifty different cyanophages infecting marine *Synechococcus* spp. and observed that, like their freshwater counterparts, they appear to belong to the same three families. However, the vast majority of marine cyanophages were Myoviridae isolates (Waterbury and Valois, 1993). They also isolated eight

morphologically different *Synechococcus* spp. cyanophages from a single water sample; five of these were isolated using the same host strain (Waterbury and Valois, 1993).

Reports from this laboratory of the isolation of marine cyanophages belonging to each of the three phage families (Wilson *et al.*, 1993; Wilson, 1994) were later shown to result from misclassifications (Wilson, personal communication). Further TEM analysis revealed that the putative Siphoviridae isolates belonged to the family Myoviridae (Wilson, personal communication). Comparison of genome sizes with those obtained in this study suggested that the tentatively-characterised Podoviridae isolate (cyanophage strain S-RSP1) also belonged to the family Myoviridae. Hence, all of the cyanophages isolated in this laboratory prior to this study were Myoviridae isolates. Suttle and Chan (1993) isolated seven cyanophages belonging to all three families, the majority being Myoviridae isolates. However, only Myoviridae isolates were found to infect phycoerythrin-containing *Synechococcus* sp. strains (Suttle and Chan, 1993).

Marine cyanophages also exhibit diversity in their host ranges. Waterbury and Valois (1993) observed that some cyanophages, which infected *Synechococcus* sp. strains from Marine-Cluster A (MC-A), *i.e.* phycoerythrin-containing strains, could infect ten different strains, whilst others could only infect a single strain. Suttle and Chan (1993) observed a cyanomyovirus which infected three phycoerythrin-containing *Synechococcus* sp. strains as well as a phycoerythrin-absent strain. Their results suggested that cyanosiphoviruses and cyanopodoviruses may be more host-specific than cyanomyoviruses (Suttle and Chan, 1993). Whilst the cyanophages isolated by Wilson *et al.* (1993) infected three different phycoerythrin-containing *Synechococcus* sp. strains, none would infect *Synechococcus* sp. WH8103 (also phycoerythrin-containing), or *Synechococcus* sp. strains PCC7002 (halotolerant, from MC-C, *i.e.* lacking phycoerythrin) or PCC7942 (freshwater), or *Anabaena* PCC7120 (a freshwater cyanobacterium). Furthermore, Waterbury and Valois (1993) observed that the freshwater *Cyanobium* PCC6307, was not infected by any marine

cyanophages. Results from host range studies therefore indicate that marine cyanophages do not infect freshwater cyanobacteria (Suttle and Chan, 1993).

Cyanophages from different oceanographic provinces have routinely been isolated using host strains from different locations. Suttle and Chan (1993) used *Synechococcus* sp. hosts from the North and tropical Atlantic to isolate cyanophages from coastal waters of Texas and New York, the Gulf of Mexico and a hypersaline lagoon. Similarly, Wilson (1994) used *Synechococcus* sp. WH7803, from the north-west Atlantic, to isolate cyanophages from Miami, Bermuda, the English Channel, the North Sea and even the Red Sea.

1.2.2 Ecological importance

Temperate cyanophages have been isolated for the freshwater cyanobacterium, *Plectonema boryanum* (Cannon *et al.*, 1971; Padan *et al.*, 1972; cited by Ohki and Fujita, 1996). However, until recently, though temperate cyanophages had been implicated, none had been isolated for marine cyanobacteria. Temperate marine viruses, including cyanophages, are discussed later (1.3.3.2).

The mechanism of resistance amongst natural *Synechococcus* spp. populations to their co-occurring cyanophages is currently unknown (Waterbury and Valois, 1993). However, it is possible that such resistance could result from lysogeny, conferring superinfection immunity; or from the absence of cyanophage receptors (due to mutation or nutritional factors, *e.g.* Xu *et al.*, 1997); or from the presence of restriction systems (for review see Carr and Mann, 1994). It is considered that cyanophages would be a major factor structuring the cyanobacterial community, affecting the clonal composition of *Synechococcus* spp. populations (Waterbury and Valois, 1993). However, it is difficult to determine the impact of cyanophages on *Synechococcus* spp. population dynamics, because of the host range diversity of cyanophages, and because of the diversity in host resistance (Suttle and Chan, 1993).

The results of Waterbury and Valois (1993) indicated that predominantly sensitive cells existed only as a minor fraction of the total *Synechococcus* spp. population, but that they were capable of out-competing the resistant cells during enrichment processes. Cyanophage receptors may commonly be involved in nutrient acquisition (Heller, 1992); hence, a mutant cyanophage receptor, rendering the cell resistant to cyanophage infection, may also have reduced efficiency of nutrient uptake, thereby reducing the growth efficiency of the cell (for review see Wilson and Mann, 1997).

Waterbury and Valois (1993) suggested that cyanophage numbers were maintained by the infection and lysis of the relatively rare sensitive *Synechococcus* spp. cells in the community. However, Suttle and Chan (1994) concluded that most *Synechococcus* spp. cells offshore were susceptible to infection by cyanophages; otherwise, calculated contact rates (Murray and Jackson, 1992) would be insufficient to produce enough cyanophages to balance their observed cyanophage decay rates. Contrary to the suggestion of Waterbury and Valois (1993), Suttle and Chan (1994) suggested that, in offshore waters, it was unlikely that a small sensitive subset of the *Synechococcus* spp. community could be responsible for most of the cyanophage population. However, in support of Waterbury and Valois (1993), the results of Suttle and Chan (1994) implied that, in inshore waters, most of the *Synechococcus* spp. cells probably were resistant to the most abundant cyanophages.

The ecological importance of marine cyanophages has been investigated in a drive to determine their contribution to the "viral loop" (Bratbak *et al.*, 1992b), converting fixed carbon from *Synechococcus* spp. to dissolved organic carbon, thereby reducing the efficiency of nutrient and energy transfer to higher trophic levels (Suttle and Chan, 1993). Viruses and carbon flow are discussed later (1.3.4.1).

Proctor and Fuhrman (1990) observed that an average of 1.5% of marine cyanobacteria contained mature cyanophage particles. Using values based on phages of the marine bacterium *Cytophaga marinoflava*, that mature phages were visible only in the latter 10% of the latent period of infection, Proctor and Fuhrman (1990) calculated that up to 30% of marine cyanobacteria may be lysed by cyanophages

daily. However, mature cyanophages are visible in the latter 50% of the latent period during infection of freshwater cyanobacteria (Padan and Shilo, 1973). Furthermore, Waterbury and Valois (1993) observed mature cyanophages in 60% of the latent period for a cyanophage infecting a marine *Synechococcus* sp.. Hence, the calculation of Proctor and Fuhrman (1990) was probably an over-estimate, and using values for cyanophages, their results would estimate that cyanophages lysed 1.5-6% of *Synechococcus* spp. daily (Suttle and Chan, 1994).

Waterbury and Valois (1993) determined *Synechococcus* spp. mortality by theoretical rates of cyanophage adsorption and measured abundances of the host and its cyanophages. They calculated that, in coastal waters, up to 3.2% of *Synechococcus* spp. populations could be lysed daily; although they also observed values as low as 0.005%. Open ocean values reached 1-2% mortality per day. They therefore suggested that lytic cyanophages would not be major contributors to *Synechococcus* spp. mortality in either coastal or oceanic waters, and that they had a negligible effect in regulating *Synechococcus* spp. population densities (Waterbury and Valois, 1993).

Suttle and Chan (1994) calculated that *ca.* 5-7% of the *Synechococcus* spp. population offshore would be lysed daily by cyanophages, but only *ca.* 0.8-5% inshore, based on measured cyanophage decay rates. Hence, with three different methods, the consensus is that cyanophages account for *ca.* 1-7% of *Synechococcus* spp. mortality in the marine environment. However, these estimates are based upon virulent cyanophages which cause irreversible lytic infections. The contribution of temperate cyanophages to the regulation of marine *Synechococcus* spp. populations is unknown. Furthermore, recent research (Wilson *et al.*, 1996) suggests that the phosphate status of *Synechococcus* spp. cells will have a profound effect on the outcome of the cyanophage-host interaction, with reference to lysis/lysogeny, and therefore affect the dynamics of carbon flow in the marine environment.

1.2.3 Molecular characterisation

Whilst the molecular characterisation of other marine viruses is being increasingly well documented (Cottrell and Suttle, 1995b; Kellogg *et al.*, 1995; Chen *et al.*, 1996), relatively little molecular characterisation of marine cyanophages has been reported.

Wilson *et al.* (1993) and Wilson (1994) reported partial molecular characterisation of six genetically distinct marine cyanophages, isolated from six different oceanographic regions. These cyanophages were later re-characterised as all being cyanomyoviruses (Wilson, personal communication). SDS-PAGE analysis of cyanophage polypeptides revealed a major protein, presumably structural, of *ca.* 55-57 kDa which was common to each cyanophage. It was noted that this major protein was much larger than that reported for freshwater cyanophages (Safferman *et al.*, 1983; Wilson *et al.*, 1993). Genome sizes were *ca.* 80-100 kb, based on restriction analysis; although genome sizes >100 kb have been calculated (see review by Carr and Mann, 1994). Resistance of cyanophage DNA (propagated in *Synechococcus* sp. WH7803) to digestion by several restriction endonucleases was observed, suggesting extensive modification of cyanophage DNA by the host (Wilson *et al.*, 1993).

Southern hybridisation revealed that all six of the cyanophages, isolated from different regions, exhibited limited homology to each other (Wilson *et al.*, 1993; Wilson, 1994). Wilson (1994) then cloned and partially sequenced the homologous region from two of the cyanophages, discovering limited homology to a structural head protein (gp20) of the coliphage T4.

Recently Ohki and Fujita (1996) partially characterised a temperate marine cyanosiphovirus, infecting *Phormidium persicinum*. The major, presumably structural protein was *ca.* 38 kDa and the genome was *ca.* 50 kb (Ohki and Fujita, 1996); both parameters being smaller than those for the previously-characterised cyanomyoviruses (Wilson *et al.*, 1993). However, in accordance with previous observations (Wilson *et al.*, 1993), this cyanophage also had DNA resistant to

restriction by some endonucleases, and was concluded to be highly methylated (Ohki and Fujita, 1996).

1.3 Marine Viruses

The existence of bacteriophages has been known for many decades (d'Herelle, 1926). However, the first report of viruses in the marine environment was not until much later (Zobell, 1946). Zobell (1946) reported the presence of an enteric phage in coastal water. However, no phages were detected in the open ocean, so the coastal isolate may have been a contaminant from sewage (Zobell, 1946). Zobell (1946) predicted that there would be no indigenous marine phages in the open oceans, since phages need high bacterial concentrations to reproduce, and the bacterial population of the oceans was generally thought to be low.

The first report of an indigenous marine virus was by Spencer (1955), who isolated a bacteriophage from a luminescent bacterium, probably *Photobacterium phosphoreum*, in the North Sea. Marine phages were subsequently defined as phages which infect a marine host, and whose reproduction and stability is dependent on salinities and temperatures usually encountered in the marine environment (Spencer, 1955; Borsheim, 1993). Whilst Spencer (1955) suggested that marine phages should have a lower heat tolerance than terrestrial phages, since marine temperatures vary comparatively little, many phages of indigenous marine bacteria have demonstrated high heat tolerance (Hidaka, 1972). Therefore, salinity is regarded as a more important determining factor. Borsheim (1993) did not include phages found in fish or shellfish as "marine", since the bacterial host was not actually growing in the marine environment.

Despite their earlier discovery, little investigation was subsequently made into marine viruses, largely because they were thought to be present in low numbers, and were therefore considered ecologically unimportant (Wiggins and Alexander, 1985).

1.3.1 Abundance and distribution

Infective techniques, such as plaque assay, revealed very low phage concentrations in the marine environment: 0-100 plaque-forming units (PFU) ml⁻¹ (e.g. Moebus, 1980), largely because they were just detecting single phage types. The first report of high abundances of marine viruses was by Torella and Morita (1979), who counted total virus abundances by transmission electron microscopy (TEM), and observed concentrations of >10⁴ virus-like particles (VLP) ml⁻¹ off the coast of Oregon, west USA. It was not until the late 1980s that very high virus concentrations were observed, at 10⁷ VLP ml⁻¹ in the north-east Atlantic and Chesapeake Bay, east USA (Bergh *et al.*, 1989); although Sieburth *et al.* (1988) and Proctor *et al.* (1988) had observed total concentrations up to 10⁶ VLP ml⁻¹. Bergh *et al.* (1989) counted the viruses directly concentrated from seawater onto a TEM grid, by ultracentrifugation, and observed much greater virus concentrations than Torella and Morita (1979). Torella and Morita (1979) only counted those viruses which collected on a 0.2 µm pore-size membrane, and it is now known that the majority of marine viruses are smaller than 200 nm (for review see Borsheim, 1993), thus passing through such a membrane. The discovery of high virus abundances sparked much interest in their anticipated ecological importance, and the amount of research into marine viruses expanded rapidly.

Virus counts from infective techniques still show only low natural concentrations. For bacteriophages these are typically 0-10 PFU ml⁻¹ per host species, although 10²-10³ PFU ml⁻¹ have been observed (Moebus, 1991; 1992b; for review see Bratbak and Heldal, 1995). For viruses which infect a eukaryotic phytoplankton, concentrations were much lower (2-700 infective units per litre; Suttle and Chan, 1995). In contrast, cyanophages which infect *Synechococcus* spp. are commonly 10⁵ infective units ml⁻¹ (Suttle and Chan, 1994). However, total virus concentrations appear to be typically *ca.* 10⁶-10⁷ VLP ml⁻¹, occasionally 10⁸ VLP ml⁻¹, depending on the season and location (e.g. Proctor and Fuhrman, 1990; Hara *et al.*, 1991; Wommack *et al.*, 1992; Weinbauer *et al.*, 1993; Jiang and Paul, 1994; Hennes and Suttle, 1995; Steward *et*

al., 1996). Concentrations can be established by TEM, or by epifluorescence microscopy of samples which have been stained with a nucleic acid dye, such as 4, 6-diamidino-2-phenylindole (DAPI) (Hara *et al.*, 1991) or the brighter dyes YOYO-1, POPO-1, *etc.* (Hirons *et al.*, 1994; Hennes and Suttle, 1995). Indeed, Hennes and Suttle (1995), using the dye YO-PRO-1, recorded virus concentrations frequently at 10^7 - 10^8 VLP ml⁻¹, and up to 10^9 VLP ml⁻¹ above a submerged cyanobacterial mat, and suggested that TEM underestimates total virus concentrations.

Both TEM and fluorescent staining of natural virus populations, will count all "virus-like" particles. The majority of these probably are viruses (Hennes and Suttle, 1995), but there is always the possibility that a VLP is not a virus. Nucleic acid dyes would be unable to distinguish between large viruses (Sommaruga *et al.*, 1995) and very small bacteria. Furthermore, these techniques cannot distinguish infective from non-infective viruses, and it may be that a large proportion of VLPs are non-infective (Suttle and Chen, 1992). In addition, little distinction can be made between virus strains. Though TEM reveals morphology, many viruses with different hosts look virtually identical (Hidaka and Fujimura, 1971; Frank and Moebus, 1987).

The method of harvesting the viruses prior to enumeration can affect the final counts. TEM and fluorescence counts are facilitated by prefiltration of the sample to remove larger particles. However, Paul *et al.* (1991) observed that such prefiltration of samples through 0.2 μ m pore-size filters reduced the viral count by, on average, two thirds, yet often by 90%. Furthermore, different filter types appear to reduce the infectivity of viruses to differing degrees, with polycarbonate filters being relatively inert (Suttle *et al.*, 1991a).

Viral abundance appears to be correlated with other biological parameters, such as bacterial numbers, chlorophyll *a*, primary production and dissolved and particulate DNA (Steward *et al.*, 1992a; Boehme *et al.*, 1993; Jiang and Paul, 1994; Maranger and Bird, 1995), suggesting that bacteria and phytoplankton are hosts of a large proportion of marine viruses. Viral abundance is therefore greatest near the coast, where nutrients and production are higher, and decreases into the oligotrophic open

ocean (Steward *et al.*, 1992a; Boehme *et al.*, 1993; Cochlan *et al.*, 1993; Jiang and Paul, 1994; Paul *et al.*, 1997). Other reports of the decrease in viral abundance offshore have observed stronger inverse correlations with salinity: as salinity increased offshore, virus abundance decreased (Paul *et al.*, 1993; Weinbauer *et al.*, 1993; Hennes and Suttle, 1995). Weinbauer *et al.* (1993) also observed a correlation between virus numbers and the trophic condition of the water in the northern Adriatic Sea. Viral counts were greatest in eutrophic waters and decreased in mesotrophic waters.

Vertically, in the water column, as expected, viral abundance is highest in the euphotic zone, and decreases below (Boehme *et al.*, 1993; Cochlan *et al.*, 1993; see review by Koike *et al.* 1993). Yet viruses appear to be ubiquitous throughout the world's oceans, and have been found at 10^5 VLP ml⁻¹ at 1500 m depth in the subtropical North Atlantic (Paul *et al.*, 1991), and even in subarctic abyssal waters at 10^5 VLP ml⁻¹ (Koike *et al.*, 1993).

Seasonal variations in virus abundance occur in temperate waters (Bergh *et al.*, 1989; Bratbak *et al.*, 1990; Smith *et al.*, 1991; Jiang and Paul, 1994; Weinbauer *et al.*, 1995). Abundance is generally low in winter months (*ca.* 10^4 - 10^5 VLP ml⁻¹), rising through spring and summer. Typically, virus concentrations in productive seasons are *ca.* 10^6 - 10^7 VLP ml⁻¹ in coastal waters, and 10^5 - 10^6 VLP ml⁻¹ in the open ocean, according to TEM (for review see Bratbak *et al.* 1994). Weinbauer *et al.* (1995) concluded that virus concentrations were greatest in summer and autumn because spring and late summer blooms of phytoplankton would release organic carbon, stimulating bacterial production, which in turn would result in greater virus production.

Marine virus to bacteria ratios (VBRs) can range from <0.1 to >50, but are usually 2-20 (Bergh *et al.*, 1989; Hara *et al.*, 1991; Wommack *et al.*, 1992; Weinbauer *et al.*, 1993; Weinbauer *et al.*, 1995); *i.e.* viruses are generally observed to be an order of magnitude more abundant than bacteria (*e.g.* Borsheim *et al.*, 1990; Steward *et al.*,

1996). However, the VBR appears to be greater in coastal and eutrophic waters than in the oligotrophic open ocean (Hara *et al.*, 1991; Weinbauer *et al.*, 1993).

1.3.2 Composition of virus communities

Bacteriophages appear to make up the majority of marine viruses (Bergh *et al.*, 1989; Wommack *et al.*, 1992; Cochlan *et al.*, 1993; Maranger *et al.*, 1994). Borsheim *et al.* (1990), Cochlan *et al.* (1993) and Weinbauer *et al.* (1993) observed that most marine viruses had head diameters <60 nm, whilst relatively few were >80 nm. Furthermore, Maranger and Bird (1995) observed that >80% of viruses were <70 nm. Capsid size, and even morphology, is not totally reliable for defining bacteriophages, since some viruses of eukaryotic phytoplankton may possess tails, and *ca.* 30% have capsids <60 nm diameter (see review by Van Etten *et al.*, 1991; Milligan and Cosper, 1994). However, correlation between viral counts and bacterial counts, and the observation of bacteria being the major host organisms (Cochlan *et al.*, 1993) suggest a predominance of bacteriophages.

However, the viral community comprises a wide range, from cyanophages infecting cyanobacteria (*e.g.* Proctor and Fuhrman, 1990), to viruses of eukaryotic phytoplankton (for reviews see Dodds, 1979; Van Etten *et al.*, 1991) and viruses of heterotrophic flagellates (Nagasaki *et al.*, 1993; Garza and Suttle, 1995). Viruses have been found which infect a wide range of marine algae, including the algal classes: Rhodophyceae; Phaeophyceae; Prasinophyceae; Prymnesiophyceae; Chrysophyceae; Chlorophyceae; and Cryptophyceae (see review by Dodds, 1979; Suttle *et al.*, 1990; Suttle *et al.*, 1991a). In particular, viruses of marine brown algae (Phaeophyceae) have been extensively studied (for review see Muller, 1996). However, much research has also been made into viruses of *Micromonas pusilla*, a photosynthetic flagellate of the class Prasinophyceae (Mayer, 1977; Mayer and Taylor, 1979; Cottrell and Suttle, 1991; 1995a; 1995b).

Marine bacteriophages have been isolated which infect several different genera, including: *Vibrio*; *Cytophaga*; *Pseudomonas*; *Achromobacter*; *Aeromonas*; *Photobacterium*; *Lucibacterium*; and *Flavobacterium* (see review by Moebus, 1987). Perhaps the most well studied of these is the genus *Vibrio*, yet this may be due largely to its relative ease of laboratory cultivation. Isolation of phages from marine waters is generally very difficult, and requires enrichment techniques and many attempts with many bacterial strains to detect any at all (Spencer, 1955; Hidaka, 1971; Moebus, 1980; see review by Borsheim, 1993). Indeed, Hidaka (1971) isolated 8 phage-host systems only after screening 68 bacterial strains, whilst Moebus (1980) found only 213 bacterial strains sensitive to phages, out of 931 isolates. Most marine phages appear to be unculturable, parallel to the problem of unculturable bacteria (see review by Borsheim, 1993).

Frank and Moebus (1987), in a study of 75 marine phages, observed that all had tails and icosahedral or octahedral heads, and were therefore similar to terrestrial phages. The phages corresponded to Bradley groups A, B and C (Bradley, 1967), now assigned to the families Myoviridae, Siphoviridae (formerly Styloviridae) and Podoviridae respectively (Matthews, 1979). However, some had knob-like projections and long spines, apparently unique to marine phages. Frank and Moebus (1987) suggested that the long spines may function to increase the probability of contacting a host cell in an environment where host concentrations are generally low. The vast majority of marine viruses appear to possess DNA rather than RNA genomes. Frank and Moebus (1987) observed that none out of 75 phages possessed RNA genomes. In addition, most viruses of marine unicellular eukaryotes preferentially have DNA genomes (see review by Van Etten *et al.*, 1991).

Attempts have been made to determine phage burst sizes, using cultured marine bacteria. A survey by Borsheim (1993) revealed an average burst size of 185 PFU cell⁻¹, although with much variation about the mean (standard deviation = 179). However, cultured cells are generally larger than those *in situ*, and may therefore give artificially high results (see review by Borsheim, 1993). Chemical lysis of bacteria *in situ*, using streptomycin, has given an average burst size of 50 phages

cell⁻¹ (Heldal and Bratbak, 1991); however, lysis may be premature, resulting in underestimates. Direct TEM analysis revealed an average burst size in a marine bacterial community of 48 (Weinbauer and Peduzzi, 1994).

1.3.3 Production and decay

1.3.3.1 Lytic production

Viral production is generally either by the lytic pathway, with infection followed by lysis; or by the lysogenic pathway, in which the viral chromosome may well be integrated into the host chromosome and replicated along with it, as a prophage. Induction of the prophage, by environmental factors, then allows it to enter the lytic pathway.

Virus infection is affected by host density. The mechanics of the system often require there to be a sufficient, threshold density of host cells for the viruses to attach before rapid viral production can proceed (Wiggins and Alexander, 1985; Suttle and Chan, 1994). Wiggins and Alexander (1985) observed thresholds of *ca.* 10⁴ bacteria ml⁻¹ for three terrestrial phage-host systems; whilst Suttle and Chan (1994) observed a threshold of *ca.* 10³ cyanobacteria ml⁻¹ for marine cyanophage replication. Wilcox and Fuhrman (1994) observed that lytic infection would only occur in coastal bacterial populations when the product of viruses and bacteria was >10¹². However, Kokjohn *et al.* (1991) observed no such threshold densities for replication of *Pseudomonas aeruginosa* phages, with attachment and replication being possible at host concentrations of 10 ml⁻¹. Weinbauer and Peduzzi (1994) observed thresholds for marine rod-shaped bacteria (10⁵ ml⁻¹), but observed no thresholds for cocci or spirillae. Hence, their results indicated that phage production is strongly influenced by the morphotypical structure of the bacterial community.

Bratbak *et al.* (1994) calculated that if bacterial diversity was high, with many different phage-host systems, then the rate of lytic infection would be low, since the host concentration for each system would be low. This coincides with the observation that, whilst total viral counts are high (*ca.* 10^7 VLP ml⁻¹) (Bergh *et al.*, 1989), concentrations of specific viruses are usually low (1-100 PFU ml⁻¹) (Moebus, 1992b). Bratbak *et al.* (1994) and Suttle *et al.* (1991b) suggest that lytic infections may be more important when specific host concentrations are high, such as in bloom periods. However, Steward *et al.* (1992a) suggest that bacteria which surround particles (*e.g.* marine snow) may be an important source of lytic virus production, since such aggregations may easily exceed the threshold host density required for rapid viral production.

Changes in abundance of marine viruses result from continual production and decay of viruses, and demonstrate that they are a dynamic component of the marine microbial community. Abundance changes on a seasonal, diel and hourly basis (Bratbak *et al.*, 1990; Heldal and Bratbak, 1991; Bratbak *et al.*, 1992b; Weinbauer *et al.*, 1995). Viral production rates are calculated to be relatively low (*ca.* 1 day⁻¹) when the sampling time is several days, yet much higher if the time is reduced to 10-15 min (*ca.* 100 day⁻¹) (see review by Bratbak *et al.*, 1994). Indeed, Bratbak *et al.* (1996a) observed a 2-4-fold increase in virus numbers *in situ* within 10-20 min, demonstrating a highly dynamic viral community. Higher production rates may correspond to simultaneous lysis events, releasing many virus particles, coupled with rapid decay rates (Bratbak *et al.*, 1996a). The lower rates may correspond to the rates of such processes as bacterial growth, grazing and nutrient turnover (see review by Bratbak *et al.*, 1994).

To obtain an estimate of the proportion of bacteria which are infected by viruses, bacteria may be thin-sectioned and examined for the presence of viruses by TEM. Proctor and Fuhrman (1990; 1991) have observed 1-10% of marine heterotrophic bacteria to contain mature virus particles, with lowest values in oligotrophic waters. Weinbauer *et al.* (1993) also observed a greater degree of infection in bacteria from eutrophic than from mesotrophic waters, suggesting that viral impact upon bacteria is

greatest in eutrophic waters. Other observations report up to 30% of bacteria containing virus particles (Heldal and Bratbak, 1991; Bratbak *et al.*, 1992b). However, mature VLPs are only visible for the last part of the lytic cycle. Proctor *et al.* (1993) calculated that the proportion of bacteria which were involved in virus production was 3.7-7.1 times that observed to contain mature VLP. However, this was based upon TEM studies of one-step growth experiments of just two marine vibriophages. Proctor and Fuhrman (1990) calculated that, on average, viruses account for 34% of bacterial mortality. Bratbak *et al.*, (1992b) calculated bacterial lysis rates, based on TEM observations. However, bacterial lysis was estimated to be six times greater than bacterial production (Bratbak *et al.*, 1992b), suggesting inaccuracies of the method; in particular, of the conversion factor between containment of mature VLPs and infection (from Proctor *et al.*, 1993).

Virus production may be assessed by the net change in viral abundance, as observed by TEM (Borsheim *et al.*, 1990). An alternative method to obtain estimates of virus production is to stop production using a metabolic inhibitor, such as cyanide, and measure viral disappearance (Heldal and Bratbak, 1991; Bratbak *et al.*, 1992b; Suttle, 1992). This method is based on the observation and assumption that viral concentrations remain fairly constant for the duration of the experiment, such that virus production rates equal virus decay rates. However, this method also assumes that cyanide does not affect viral decay, and Suttle and Chen (1992) observed that cyanide actually decreases decay rates, suggesting that biological processes are involved. Hence, this is not an accurate method for measuring viral production.

More accurate methods for estimating viral production appear to be by incorporation of ^3H -thymidine and ^{32}P -orthophosphate (Steward *et al.*, 1992a; 1992b). Steward *et al.* (1992a; 1992b) observed that virus production rates were *ca.* 30 times greater for coastal, compared with offshore regions. Using the same method, in Arctic seas, Steward *et al.* (1996) observed that viruses were responsible for, on average, *ca.* 23% of bacterial mortality, with individual samples reaching 36%. The results, using different techniques to measure virus production, are relatively consistent, suggesting

that on average 20% of marine heterotrophic bacteria are infected, and that 10-20% of the bacterial community is lysed daily by viruses (for review see Suttle, 1994).

1.3.3.2 Lysogeny

Freifelder (1987) observed that >90% of all known phages appeared to be temperate. It is generally considered that most marine viruses are temperate, able to survive long periods by lysogenising their host, although there is little hard evidence to prove this (for reviews see Bratbak *et al.*, 1993b; Wilson and Mann, 1997). Indeed, in a study by Moebus (1983), out of 300 marine phages tested, only 29 were found to be temperate. However, lysogeny is expected to be common and favoured above lysis for survival, since marine host concentrations are often low and decay rates would be high for free virus particles (see review by Bratbak *et al.*, 1994). Most viral production would therefore be a result of induction from lysogeny rather than new infection, and hence depend upon environmental triggers rather than virus-host dynamics. However, the results of Wilcox and Fuhrman (1994) suggested that most of the phage production which they observed in Californian coastal waters was from lytic infection rather than induction from lysogeny. Furthermore, Weinbauer and Suttle (1996) concluded from their observations that induction from lysogeny was generally not an important source of phage production in coastal waters of the Gulf of Mexico.

Observations of high virus production rates over short time periods may be due to synchronous lysis of a large part of the microbial population. Indeed, the sudden collapse of blooms, associated with virus production, has been observed for the coccolithophore *Emiliana huxleyi* (Bratbak *et al.*, 1993a; Bratbak *et al.*, 1996b; Brussaard *et al.*, 1996b; Wilson *et al.*, 1997) and the red tide alga *Heterosigma akashiwo* (Nagasaki *et al.*, 1994). Such large lysis events may result from simultaneous induction of many lysogenic hosts (see review by Bratbak and Haldal, 1995). Hence, lysogeny may be important for viruses of marine eukaryotes as well as for marine bacteriophages (see review by Wilson and Mann, 1997).

Evidence for lysogeny is also supported by Jiang and Paul (1994), who induced virus production in 43% of cultured marine bacteria. However, their results may not be representative of the overall system, since they only screened those phage-host systems which could be isolated. Weinbauer and Suttle (1996) estimated that only 0.07-4.4% of marine bacteria were lysogenised, and found no difference in the occurrence of lysogeny between oligotrophic and nutrient-rich waters, using mitomycin C and UV-C to induce lysogens. However, Jiang and Paul (1996) used additional agents to induce lysogens (mitomycin C, UV, temperature, pressure and polyaromatic and aliphatic hydrocarbons), and accordingly observed a higher incidence of lysogeny (up to 38%). Ohki and Fujita (1995; 1996) recently isolated a temperate cyanophage which lysogenises the marine cyanobacterium *Phormidium persicinum*, and more recently a temperate cyanophage of marine *Synechococcus* sp. NKBG042902 has been isolated (Sode *et al.*, 1997).

The rate of viral production by induction from lysogeny depends on certain environmental factors. Several factors are known to cause induction in the laboratory; in particular UV light and mitomycin C. Loessner *et al.* (1991) discovered that mitomycin C was a more powerful inducing agent than UV light for *Listeria* spp. lysogens. Weinbauer and Suttle (1996) observed a similar result for marine bacteria. Furthermore, induction of a marine cyanophage has recently been shown using Cu^{2+} , although other heavy metals were not effective inducers (Sode *et al.*, 1997). However, the precise triggers *in situ* are unknown. UV light is an unlikely candidate, since direct sunlight was found to have no effect on virus production in coastal seawater (Wilcox and Fuhrman, 1994), and it is not regarded that temperature change would be an important factor for induction in marine systems (see review by Wilson and Mann, 1997). Traditionally it has been thought that nutritional conditions and multiplicity of infection (MOI) are the main factors in determining induction in the natural environment (Freifelder, 1987). Usually low nutrient availability and high MOI favour lysogeny (Wilson *et al.*, 1996; see review by Wilson and Mann, 1997). Generally, within a lysogenic population a small proportion of cells are continually induced and lysed, so that a small number of free viruses will also be present (see review by Bratbak *et al.*, 1994).

Jiang and Paul (1996) observed more induction in natural populations of coastal bacteria than offshore bacteria. These results contradict their previous findings, based on cultivable bacteria, of more lysogens in oligotrophic offshore waters than nutrient-rich coastal waters (Jiang and Paul, 1994). However, Wilson *et al.* (1996) observed vastly reduced lysis of cyanophage-infected *Synechococcus* sp. which had been cultured in phosphate-deplete, compared with phosphate-replete medium; suggesting lysogeny under conditions of low phosphate availability. Furthermore, Bratbak *et al.* (1993a) observed inhibition of virus production in *Emiliana huxleyi* under phosphate, but not nitrate, limitation, perhaps because viruses have a high nucleic acid to protein ratio. Induction of lysogens has also been implicated when increase in nutrient availability has brought a subsequent collapse in host populations (Tuomi *et al.*, 1995; Wilson *et al.*, 1997). These results suggest a dependence of lysogeny upon environmental nutrient conditions. In contrast, Wikner *et al.* (1993) showed that three marine phages obtained most of their nucleotides from the host nucleic acid, and suggested that this may be a common adaptation to the general nutrient deficiency of the marine environment.

Pseudolysogeny is a form of phage-host interaction which has recently been observed in marine systems (Moebus, 1997a; 1997b; 1997c). This situation arises when the infected host is starved, and there is insufficient energy either for a lytic or a true lysogenic response. Hence, the phage DNA remains inactive and unintegrated. However, an increase in nutrients will bring about either lysis or lysogeny. It is possible that pseudolysogeny may explain the long-term survival of viruses in oligotrophic waters (Ripp and Miller, 1997).

There is, as yet, no direct evidence to suggest that MOI affects lysogeny amongst marine viruses. However, the VBR is greater in eutrophic waters than oligotrophic waters (see review by Bratbak and Haldal, 1995). Whilst MOI is not necessarily determined by the VBR, this suggests that there would be a higher MOI in eutrophic waters, favouring lysogeny, as observed by Jiang and Paul (1996). Waterbury and Valois (1993) observed that a large proportion of *Synechococcus* sp. cells were resistant to their co-occurring cyanophages. It is known that lysogeny gives the cell

immunity to viruses of the same type as has lysogenised the cell. Hence, the observed resistance could be a result of such superinfection immunity, suggesting that most of the *Synechococcus* spp. population is lysogenic.

1.3.3.3 Decay

Decay is an important factor for viruses in the marine environment, since host concentrations are generally low, and it may be a considerable time between release by lysis and reinfection. Early investigation into decay of viruses in seawater was directed towards the inactivation of pathogenic enteric phages which contaminate coastal waters (for review see Kapuscinski and Mitchell, 1980). However, the majority of principles are the same for indigenous marine viruses. Inactivation was determined by a loss of plaquing ability, and was assigned either to: capsid damage, losing the ability to attach or infect; or to nucleic acid damage, losing the ability to replicate. Decay may also be defined by the loss of integrity of the virus particle, as observed by TEM. However, measurement of the loss of infectivity will give maximum decay rates, since infectivity may be lost whilst still retaining integrity.

Direct measurements of decay of natural virus populations *in situ* can only be performed by techniques which analyse the total virus population, such as TEM; hence, only a loss of integrity can be observed, and not infectivity. Infectivity is destroyed more quickly than viral integrity (Bratbak *et al.*, 1990; Wommack *et al.*, 1996); hence, a large proportion of viruses observed in seawater may not be infective (Suttle and Chen, 1992). Virucidal activity of seawater is due to a mixture of physical, biological and chemical factors.

1.3.3.3.1 Physical factors

A major cause of virus inactivation is sunlight, especially UV light, which may damage either the protein capsid or the nucleic acid (Suttle and Chen, 1992; Suttle *et al.*, 1993; Fuhrman *et al.*, 1993b; Murray and Jackson, 1993; Cottrell and Suttle, 1995a; Noble and Fuhrman, 1997). Suttle and Chen (1992) observed for marine phages that, whilst most of the decay from sunlight was attributed to UV-B, sunlight without UV-B still greatly affected viral decay. Noble and Fuhrman (1997) observed that sunlight accounted for a quarter to a third of the decay rates of native viruses, and that UV-B was particularly important in the decay. Suttle and Chen (1992) calculated that in the surface 200 m of clear oceanic waters most of the viral decay would result from sunlight, although Murray and Jackson (1993) suggest that attenuation would make UV light insignificant in viral decay below the mixed layer. Since oceanic waters contain less organic material than coastal waters, the relative importance of sunlight in viral decay would likely be greater in the more oligotrophic waters (Suttle and Chen, 1992), especially if biological material absorbs UV light, giving some protection for the viruses (Murray and Jackson, 1993). In support of this, Wilhelm *et al.* (1997) observed significantly greater damage to viral DNA from sunlight in offshore, compared with coastal waters. Viral survival may be affected by the time of release by lysis, since those released during the day near the surface, subjected to high sunlight fluxes, may decay more rapidly than those released in the evening or at night (Murray and Jackson, 1993).

Murray and Jackson (1993) suggested that UV light may be particularly important in viral decay in polar regions, where ozone depletion permits greater penetration of UV light, and sea ice is relatively transparent to UV light. In near-surface and shallow coastal waters, sunlight can destroy the infectivity of most of the viral community in just a few hours (Suttle and Chen, 1992). This may explain why infective viruses for a given bacterial host are so low (see review by Bratbak and Heldal, 1995). However, the fact that some infective viruses occur in very high concentrations in surface waters (Suttle and Chan, 1994) would seem paradoxical. In sunlight, viral infectivity is destroyed much more rapidly than viral particles (Suttle *et al.*, 1993; Wommack *et*

al., 1996). However, in deeper waters, with less light, the difference is not so great (Wommack *et al.*, 1996). Hence, in deeper waters the proportion of infective viruses, compared with total counts, should be greater (Wommack *et al.*, 1996).

Recent reports have demonstrated the repair of viral DNA which has been damaged by sunlight (Weinbauer *et al.*, 1997; Wilhelm *et al.*, 1997). Wilhelm *et al.* (1997) observed that photodamaged viral DNA was repaired at night through host-mediated mechanisms. Furthermore, Furuta *et al.* (1997) recently reported the presence in *Chlorella* viruses of a homologue of the T4 *denV* gene. In T4 this gene encodes an enzyme which repairs UV-damaged DNA. Weinbauer *et al.* (1997) reported both DNA repair and restoration of viral infectivity to sunlight-damaged viruses. Infectivity was found to be restored by the activity of blue light (370-550 nm wavelength), implying the involvement of the host-mediated phenomenon, photoreactivation (Weinbauer *et al.*, 1997). Photoreactivation repairs sunlight-damaged viral DNA once the DNA has been injected inside the host cell, using host photolyase enzymes (Bernstein, 1981). Hence, the virus can replicate and lyse the host cell. Photoreactivation, and light-independent DNA repair, may therefore partly explain the presence of high concentrations of infective viruses in surface waters where sunlight fluxes are high (Suttle *et al.*, 1993; Weinbauer *et al.*, 1997).

Different viruses decay at different rates (Suttle and Chen, 1992) and appear to be affected by sunlight to differing degrees (Noble and Fuhrman, 1997). Wommack *et al.* (1996) distinguished that two marine phages subjected to sunlight had different rates of decay of infectivity, but similar rates of capsid destruction. Noble and Fuhrman (1997) observed that viruses native to the Californian coast decayed less than those from colder regions (the North Sea) when subjected to sunlight, suggesting an adaptation of viruses native to more tropical waters to the higher sunlight fluxes.

Loss of infectivity may result from adsorption to metabolically inactive host cells, preventing lytic infection (see review by Moebus, 1987). Adsorption to larger particles, or to host or non-host organisms which then sink out of the euphotic zone

or are grazed by zooplankton, may also be a factor in viral loss from the surface waters (Suttle and Chen, 1992). Indeed, Proctor and Fuhrman (1991) have observed large numbers of viruses associated with material in sediment traps, even down to 400 m depth. However, Hennes *et al.* (1995), using fluorescently-labelled viruses as probes, did not observe any non-specific adsorption of their viruses to other particles or cells. A possible loss of overall viral infectivity may result from the coagulation of virus particles, since the viruses in the centre of an aggregate would not be able to cause an infection. Many of the viruses collected in the sediment traps were reported to be aggregated (Proctor and Fuhrman, 1991). However, using a mathematical model, Grant (1994) predicted that viral coagulation from Brownian motion would be negligible in most aquatic systems.

The survival of viruses may be prolonged, however, by reversible adsorption to colloids and particulate matter (Bitton and Mitchell, 1974). Bitton and Mitchell (1974) observed that the addition of the clay mineral montmorillonite, or non-host *Escherichia coli* cells inhibited decay of the coliphage T7. Such adsorption resulted in a decrease in plaque formation, but the virus still retained its infectivity (Carlson *et al.*, 1968).

Temperature has been investigated as a potential virucidal factor. However, many cultured marine phages were unaffected for infectivity by incubation at 50°C (Hidaka, 1972), so it is unlikely that environmentally relevant temperatures would cause virus inactivation. However, Berry and Noton (1976) observed that decay of the coliphage T2 in seawater was temperature-dependent; and Moebus (1992a) observed that some marine phages decayed faster at 20°C than at 5°C. Furthermore, Mathias *et al.* (1995) observed that decay of freshwater viruses was greater at 18-25°C than at 5-15°C.

1.3.3.2 Biological factors

Sterilisation of seawater by autoclaving or filtration through 0.2 μm pore-size filters generally reduces antiviral activity considerably (Berry and Noton, 1976; Suttle and Chen, 1992; Noble and Fuhrman, 1997), suggesting that a large amount of decay is due to heat-labile substances and filterable particles. However, Suttle and Chen (1992) observed that cyanide did not greatly reduce decay rates in three marine phages. Murray and Jackson (1992) observed that virus inactivation is positively correlated with bacterial population density, and concluded that non-host bacteria were a major cause of viral mortality. Mathematical models suggested that transport and adsorption of the viruses to bacteria could be the mechanism involved (Murray and Jackson, 1992). Furthermore, Moebus (1992a) observed that indigenous marine bacteria were important in phage inactivation, although the mechanism was unknown. However, bacterial death resulted in increased phage inactivation, presumably from the release of proteases and nucleases (Moebus, 1992a). Furthermore, Noble and Fuhrman (1997) showed that one fifth of decay rates for native marine phages were attributed to heat-labile dissolved material, >30 kDa, which were probably extracellular enzymes.

However, Suttle and Chen (1992) concluded that most bacteria were not responsible for the decay of three marine phages, since filtration through 0.8 μm or 1 μm pore-size filters greatly reduced decay rates, but not bacterial numbers. They also discovered that the 3-8 μm fraction caused viral decay, implying consumption by nanoflagellates. Heterotrophic nanoflagellates have since been shown to graze viruses directly (Gonzalez and Suttle, 1993). Murray (1995) speculated that phytoplankton might even exploit flagellate-grazing on viruses to protect themselves from viruses. He noted that healthy phytoplankton exude dissolved organic matter (Bjornsen, 1988; cited by Murray, 1995), and suggested that this supported bacterial growth, which in turn supported flagellate growth; the latter then grazing viruses (Murray, 1995). However, grazing of viruses by flagellates appears to be relatively unimportant in viral loss (Gonzalez and Suttle, 1993).

Lo *et al.* (1976) observed that virus survival was longer in winter than summer. This observation would be expected, since biological activity and sunlight intensity would be lower in the winter. Furthermore, virus decay rates measured in Californian waters are generally lower than Norwegian measurements, possibly due to the relatively oligotrophic nature of Californian waters (see Fuhrman *et al.*, 1993b). Whether the virus is temperate or lytic may also affect loss of infectivity. Moebus (1987) observed a very rapid loss of infectivity for temperate virus stocks, in comparison with lytic virus stocks. Lytic virus stocks suffered little loss even up to 7 years, if stored at 8°C (Moebus, 1987).

1.3.3.3.3 Chemical factors

Toranzo *et al.* (1981) noticed inactivation of poliovirus, probably from antibiotics produced by marine bacteria, in seawater which had been filtered, though not when it had been autoclaved. Furthermore, lipophilic and hydrophilic extracts from a whole range of cyanobacteria have been shown to inactivate the pathogenic enveloped viruses, HIV-1, HSV-2 and RSV (Patterson *et al.*, 1993). Mitchell and Jannasch (1969) also observed virucidal activity from a filterable agent, which was suggested to be heavy metals.

Ionic concentration can affect virus stability, with a decrease in salinity causing increased inactivation; indeed marine phage activity was observed to decrease to <1% after 2 h at 4°C in distilled water, or in solutions containing 15 mM Mg²⁺ and Ca²⁺ (Keynan *et al.*, 1974; Zachary, 1976; cited by Moebus, 1987). Marine viruses are also dependent upon the ionic environment for production. The concentrations of Mg²⁺, and to a lesser extent Na⁺ ions are important for adsorption of the virus to its host. Inhibition of adsorption can occur as a result of too high or too low ionic concentrations (Zachary, 1976). Ionic concentration can also affect the burst size and latent period of viruses (Zachary, 1976).

Whilst virus decay appears to be affected by sunlight, particles $>0.2\ \mu\text{m}$ and dissolved heat-labile substances, in the absence of all the above factors there still appears to be a background level of decay which is unaccounted for (Noble and Fuhrman, 1997).

1.3.4 Ecological significance

Initial interest was placed in the roles of marine viruses, largely because it was considered that they may be important in the control of host population density and diversity, accounting for an otherwise unexplainably high bacterial mortality. Viruses have been suggested to have a "side-in" control on host populations (see review by Bratbak *et al.*, 1993b), as opposed to "top-down" from grazers or "bottom-up" from nutrients. However, the most fundamental implication of high virus concentrations is the possibility of genetic exchange between hosts via transduction, as a consequence of lysogeny. Such genetic exchange within a population should permit microbial assemblages to adapt more rapidly to changing environmental conditions (as suggested by Sherr, 1989).

1.3.4.1 Viruses and the microbial loop

Viruses do not add any new processes or connections to the microbial food web, but may alter the relative importance of particulate and dissolved production at each trophic level, as lysis creates dissolved organic carbon (DOC) from particulate organic carbon (POC) (see review by Bratbak *et al.*, 1994).

A simplified account of the effects of viruses on the microbial loop has been illustrated by Bratbak *et al.* (1994) (Fig. 1.3). In the microbial loop carbon is fixed photoautotrophically by phytoplankton. Some of this fixed carbon is excreted into the DOC and colloids pool, which is then assimilated by heterotrophic bacteria.

Heterotrophic nanoflagellates (HNFs) then graze the bacteria, and are themselves grazed by zooplankton. Examples of this microbial loop were observed in bacterial peaks and increases in HNFs following diatom blooms (Bratbak *et al.*, 1990; Peduzzi and Weinbauer, 1993). In addition, HNFs have been shown to graze on viruses (Gonzalez and Suttle, 1993; Peduzzi and Weinbauer, 1993). Indeed, viruses may be nutritionally important for HNFs (Gonzalez and Suttle, 1993). Hence, DOC from phytoplankton, be it only a small proportion, is channelled back into the metazoan grazing food web.

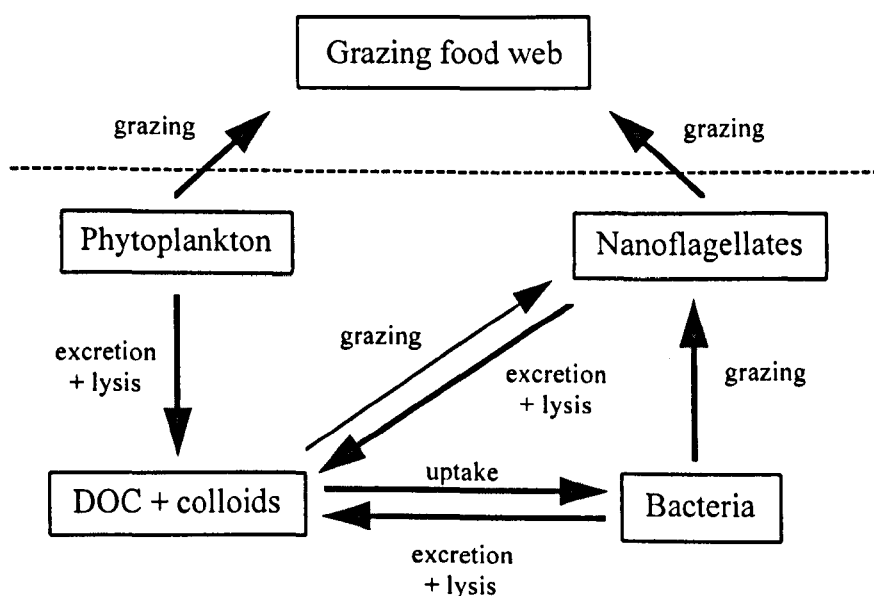


Figure 1.3 The microbial loop and the incorporation of viral activity therein. Viruses divert the fixed carbon from each trophic stage back into the dissolved organic carbon (DOC) pool via cell lysis. Modified from Bratbak *et al.* (1994).

However, DOC is released from bacteria and nanoflagellates by excretion and viral lysis, via the "viral loop" (Bratbak *et al.*, 1992b) back into the DOC and colloids pool (see review by Fuhrman, 1992). Indeed, viruses themselves constitute a proportion of the DOC, since they pass through a GF/F glass fibre filter, typically with a pore size of 0.7 μm . However, in a study of small (<120 nm) colloids, Wells and Goldberg (1991) observed that only a fraction of such were viruses, although small colloids may account for up to 10% of DOC.

Viral lysis would prevent the fixed carbon from reaching higher trophic levels, and yet sustain high bacterial activity by replenishing the DOC pool (Bratbak *et al.*, 1990; Van Boekel *et al.*, 1992). Indeed, Van Boekel *et al.* (1992) observed that lysis of a *Phaeocystis* sp. bloom (by unspecified mechanisms), releasing DOC, resulted in a corresponding increase in bacterial and microzooplankton numbers. Brussaard *et al.* (1996b) observed that viral lysis of *Emiliana huxleyi* was an important source of organic carbon for bacteria. Middelboe *et al.* (1996) added *Vibrio* sp. to a natural bacterial community, and observed that viral lysis of *Vibrio* sp. caused an increase in metabolic activity and cell production by noninfected bacteria. They suggested that lytic release of phosphate stimulated increased bacterial production. Middelboe *et al.* (1996) also observed that noninfected bacterial growth efficiency decreased as a result of *Vibrio* sp. lysis, perhaps because of the increased bacterial energy demand to degrade polymeric organic nitrogen and phosphorus in cell lysates. They suggested that viral-induced lysis may result in periodic uncoupling between DOC turnover and biomass production, and in increased energy consumption (Middelboe *et al.*, 1996). Viruses may also affect the chemical composition of dissolved organic matter (DOM), in particular dissolved amino acids and carbohydrates, as a result of cell lysis (Weinbauer and Peduzzi, 1995a).

Viruses may also affect the removal of fixed carbon into the deep ocean, by lysing larger cells which would otherwise sink out of the euphotic zone. Hence, viral lysis would help to retain fixed carbon in the surface waters (see review by Thingstad *et al.*, 1993). Viruses which infect larger phytoplankton ($>10\ \mu\text{m}$) may be especially important, since such phytoplankton appear to be particularly important contributors to particle flux out of surface waters (Michaels and Silver, 1988). Viruses themselves only account for a small proportion of the total organic carbon, and their biomass is small compared with bacteria. A liberal estimate, based on an average virus diameter of 70 nm, suggested that 10^7 viruses ml^{-1} would account for $0.27\ \text{ng C ml}^{-1}$ (Borsheim, 1993). Bacterial biomass, however, in oligotrophic regions is usually 2-40 ng C ml^{-1} , from *ca.* 10^6 bacteria ml^{-1} (Borsheim, 1990).

1.3.4.2 Dissolved DNA

Viruses contribute dissolved DNA (D-DNA) to the marine pool via host lysis and viral disintegration (Karl and Bailiff, 1989). Viruses themselves are included as D-DNA, passing through a 0.2 μm pore-size filter. Whilst D-DNA which is >30 nm and non-DNase-digestible has been observed to account for *ca.* 80% of total D-DNA (Maruyama and Higashihara, 1993), viruses themselves have been observed to contribute 1-12% of total D-DNA in seawater (Paul *et al.*, 1991). Weinbauer *et al.* (1995) observed that viral DNA contributed, on average, 18% of total D-DNA, although the range was from as little as 0.1% to as much as 96%, being higher in eutrophic waters. However, the greatest percentages occurred when total D-DNA concentrations were low, yet virus concentrations were high. Jiang and Paul (1994) observed that D-DNA varied on a diel rhythm, whereas viruses did not, and concluded that viruses constituted a small fraction of total D-DNA. Later, Jiang and Paul (1995) calculated that viruses accounted for about 8-15% of D-DNA, and showed, using kingdom probing based on rRNA-specific probes, that most D-DNA was from eubacteria and eukaryotes rather than viruses.

Viruses indirectly add to the D-DNA pool through host lysis, releasing D-DNA. Weinbauer *et al.* (1995) estimated that viral-mediated bacterial lysis may account for up to 82% of total D-DNA in surface waters. About 26-38% of the oceanic DNA pool is accounted for by D-DNA, the remainder being particulate, *i.e.* picoplankton and larger plankton (Jiang and Paul, 1995); however, D-DNA is usually $<10\%$ of total dissolved organic phosphate (DOP) (Paul *et al.*, 1988). Therefore, viral contribution to oceanic DNA is minimal (2-6%)(Jiang and Paul, 1995), as it is to DOP, though viral contribution is greater in coastal and eutrophic waters than in the oligotrophic open ocean (Paul *et al.*, 1991; Weinbauer *et al.*, 1993). However, the rapid turnover rates, observed by production and decay, may mean that viruses are important in phosphate cycling, especially in the oligotrophic open ocean.

1.3.4.3 Impact upon host populations and processes

The effects of viruses on the diversity of a microbial community *in situ* are difficult to prove. However, Bratbak *et al.* (1990) showed that viral lysis may change the composition of a bacterial community growing in the mucous which surrounds diatoms. Furthermore, the viral-induced collapse of *Emiliana huxleyi* blooms has allowed other phytoplankton species to bloom (Bratbak *et al.*, 1993a). Viruses are able to exert a species-specific control, and may allow high host diversity and the coexistence of competing species by causing the collapse of any dominant blooms (see review by Thingstad *et al.*, 1993). Indeed, Hennes *et al.* (1995) showed that viruses specific for *Vibrio natriegens* could control host populations even from an undetectable background concentration, observing a collapse of host numbers with a corresponding increase of the phage from <1 PFU ml⁻¹ to $>10^7$ PFU ml⁻¹. Viruses may therefore have a considerable influence on the structure of the microbial food web. Viral control of the host would be highly specific if resulting from specific lytic infections, but more general if resulting from induction of lysogens by environmental factors. Hence, lysis should have a different effect on the food web dynamics from non-specific grazing (for review see Thingstad *et al.*, 1993).

Viruses may be important in influencing the dynamics and oscillation patterns in plankton community succession. Peduzzi and Weinbauer (1993) observed that enrichment of the virus fraction of seawater by 2.5 times delayed the onset of a diatom bloom; however, the final diatom biomass was slightly greater than the control. They also noticed correspondingly more pronounced peaks in heterotrophic microbe populations. The viral peak coincided with high bacterial numbers, suggesting a close coupling between viruses and bacteria. In addition, a decrease in bacterial and viral numbers coincided with an increase in HNFs, suggesting grazing on bacteria and viruses.

Weinbauer and Peduzzi (1995b) observed *in situ* diel variability of bacterial and viral counts in the northern Adriatic Sea, showing predator-prey dynamics over time-scales of hours. Viral abundance was more closely coupled to bacterial numbers than

was HNF abundance. This would suggest that, in such mesotrophic and eutrophic waters, viruses are perhaps more important in bacterial control than HNFs, diverting POC back to DOC (Weinbauer and Peduzzi, 1995b). Their data suggest that viral lysis is more important in bacterial mortality in more eutrophic waters (Weinbauer and Peduzzi, 1995b). However, Steward *et al.* (1996) observed that bacterial mortality in Arctic seas by viral lysis was about equal to that from flagellate grazing. Fuhrman and Noble (1995) reported similar observations in bacterial mortality in Californian coastal waters.

Studies on the effects of viruses upon phytoplankton have shown vast decreases in primary productivity following the addition of concentrated viral fraction to seawater (Suttle *et al.*, 1990; Suttle, 1992). Oloffson and Kjelleberg (1991) argued that this would not be a sustained effect, due to the strong selection for virus-resistant phytoplankton cells. However, Suttle *et al.* (1991b) responded that viruses may have a considerable impact upon monospecific phytoplankton blooms, and a smaller, yet still evident, impact upon non-blooming phytoplankton populations. Viral infections in marine brown algae have been observed to reduce photosynthesis and concentrations of chlorophylls *a* and *c* (Robledo *et al.*, 1994).

The discovery of a virus infecting the marine prymnesiophyte *Phaeocystis pouchetii* (Jacobsen *et al.*, 1996) has important ecological implications. This photosynthetic flagellate forms dense, nearly monospecific blooms and produces dimethylsulphoniopropionate (DMSP), which, via conversion to DMS, may be important in climate control (see section 1.1) (for review see Liss *et al.*, 1994). Recent research has demonstrated viral-mediated release of DMSP from *Phaeocystis pouchetii* (Malin *et al.*, submitted). Efforts to identify a relationship between viral lysis of the globally important *Emiliana huxleyi* and DMS release have failed, probably due to the relatively low density of *E. huxleyi* blooms, which only reach 10^4 cells ml⁻¹ (Bratbak *et al.*, 1995).

Murray and Eldridge (1994) constructed a mathematical model to estimate the effects of the microbial loop on the metazoan food web. They suggested that the microbial

loop is unlikely to supply much nutrition to mesozooplankton (*i.e.* plankton 20-200 μm), and therefore to the metazoan food web, in mesotrophic coastal waters, since the microbial loop is very inefficient, losing energy at each trophic stage. However, in oligotrophic waters, where bacteria are grazed directly by mesozooplankton, such as salps, the microbial loop is likely to be more important on the metazoan food web. Hence, bacteriophages would have a greater impact upon mesozooplankton in oligotrophic waters. High levels of phage activity can decrease zooplankton production by as much as 5-15%, thus considerably affecting higher trophic levels (Murray and Eldridge, 1994).

The quantitative importance of viruses in the control of host populations *in situ* is uncertain. However, as mentioned earlier (1.3.3.1), *ca.* 10-20% of the bacterial population is lysed daily by phages (for review see Suttle, 1994). Furthermore, laboratory experiments have shown that the presence of infective phages may keep the bacterial population at 2-4 orders of magnitude lower than its potential in the absence of phages (Lenski, 1988; Miller and Saylor, 1992; cited by Bratbak *et al.*, 1994). Production of viruses which infect phytoplankton can be assessed by the suppression of photosynthesis (Suttle *et al.*, 1990; Suttle, 1992), and experiments suggest that eukaryotic phytoplankton production would be *ca.* 2% higher in the absence of viruses (see review by Suttle, 1994). Overall, *ca.* 2-3% of marine primary production may be lost to viral lysis, although there is much variation about this mean (for review see Suttle, 1994). The greatest impact of marine viruses, however, is expected to be through maintenance of species diversity and genetic exchange.

Whilst total virus populations can be examined for their ecological significance, great difficulty exists in investigating the relationship between a specific host and its virus population in a mixed natural community. The problem is less for phytoplankton and flagellates, since morphological differences can be observed by microscopy. Many bacteria, however, appear morphologically identical. In addition, whilst some viruses can be distinguished morphologically (*e.g.* by unusually large size), and therefore traced in the environment (Bratbak *et al.*, 1992a; Bratbak *et al.*, 1993a; Gowing, 1993), many viruses also appear identical (Hidaka and Fujimura, 1971; Frank and

Moebus, 1987). This problem can be partially surmounted by fluorescent tagging of specific viruses and their release and observation in natural systems (Hennes *et al.*, 1995).

1.3.4.4 Genetic exchange

Horizontal gene transfer by transduction occurs naturally in bacterial communities (*e.g.* Coughter and Stewart, 1989). Hence, genetic exchange by transduction may well be common among marine bacteria. However, evidence of transduction in the marine environment is minimal (Chiura and Takigi, 1994; Chiura and Kato, 1996); although lysogeny is increasingly being found (Jiang and Paul, 1994; Ohki and Fujita, 1995; Weinbauer and Suttle, 1996).

Transduction would generally be between host strains of the same genus. Borsheim (1993) observed that, of all the marine phages and hosts which have been screened, no phages infected a different host species; however, many phages infected different strains of the same species. However, Suttle and Chan (1995) observed that a virus which infected the marine prymnesiophyte *Chrysochromulina brevifilum* also lysed *C. strobilis*; while Muller *et al.* (1996) observed that a virus which infected the marine brown alga *Ectocarpus fasciculatus* also infected *E. siliculosus*, though the progeny viruses were not infective. Furthermore, Muller and Parodi (1993) observed that a virus of the marine brown alga *E. siliculosus* could infect *Feldmannia simplex* (another brown alga); though it was unable to produce infectious progeny viruses. These results therefore raise the possibility of gene transfer not only between species, but also between genera of algae, by transduction.

Bratbak and Heldal (1995) estimated that 0.36-360 of the bacteria in a community of 10^6 ml⁻¹ may be transduced daily. About 2-5% of these possible transductants may become recombinants (Birge, 1981; Weinstock, 1991; cited by Bratbak and Heldal, 1995). An important condition for transduction is that the phage must encode nucleases which fragment the host DNA, but which do not degrade it. However, it

has been observed that three marine phages obtained most of their nucleotides from host DNA degradation (Wikner *et al.*, 1993). If this is true of most marine viruses then transduction would be much less common (for review see Bratbak and Haldal, 1995). However, it is hypothesised that genetic exchange between marine viruses may occur when multiple viruses infect a host cell (Mann, personal communication). After injection of viral DNA there is a possibility of recombination between different viral DNAs before replication (for review see Cann, 1993).

1.4 Marine Microbiology

1.4.1 The microbial loop

Marine planktonic organisms can be characterised according to their size (for review see Lalli and Parsons, 1993). Microplankton (20-200 μm) and nanoplankton (2.0-20 μm) include eukaryotic algae (see below) and heterotrophic protozoa. Picoplankton (0.2-2.0 μm) comprises largely bacteria and cyanobacteria. Femtoplankton (0.02-0.2 μm) comprises largely viruses.

Azam *et al.* (1983) described the importance of marine microbes, presenting a "microbial loop" model to illustrate their ecological roles (see Fig. 1.3). The larger eukaryotic phytoplankton are grazed by microzooplankton, which then pass the fixed carbon through, eventually to fish. However, a proportion of fixed carbon is exuded from phytoplankton as dissolved organic matter (DOM), and therefore cannot be directly utilised by zooplankton. Phytoplankton cell lysis also releases DOM. Heterotrophic bacteria, with a large surface-to-volume ratio, are able to rapidly absorb and assimilate these nutrients; indeed, such bacterial growth has been positively correlated with phytoplankton cell lysis (Brussaard *et al.*, 1995). These bacteria are then grazed by heterotrophic nanoflagellates (HNFs). HNFs are then consumed by microzooplankton. Hence, the DOM exuded from phytoplankton is

recycled, with carbon flowing from phytoplankton through bacteria and HNFs, being made available for the metazoan food chain.

However, at each trophic stage in the microbial loop, a substantial amount of energy will be lost, and only a small proportion of the original production will therefore reach the higher trophic levels. From measurements of primary production and respiration Hagstrom *et al.* (1988) estimated that only 6% of production reached trophic levels higher than protozoa. Hence, the importance of the microbial loop in providing energy for metazoans is questionable.

The microbial loop is now known to be more complex than was first suggested. Nanoflagellates, which graze bacteria, also release inorganic nutrients by "sloppy feeding", recycling them for phytoplankton (Bratbak, 1987). Hence, the reserves of DOM originate from not only phytoplankton exudation and microbial excretion. Furthermore, it has been observed that nanoflagellates can by-pass bacteria, thereby creating a short-cut in the food chain, by feeding on aggregates of partially degraded phytoplankton detritus (Van Wambeke, 1994), or by feeding directly on picophytoplankton (*e.g.* Liu *et al.*, 1995).

Organisms within the marine microbial food web may be important in removing biogenic carbon from the surface of the oceans, thus preventing it from entering the metazoan food chain (for review see Legendre and Le Fevre, 1995). This may be achieved, for example, by coccolithophores (eukaryotic algae) and foraminiferans (amoebas), which contain large amounts of calcium carbonate. Sedimentation of these organisms removes fixed carbon from the system. Carbon is also removed by sedimentation of marine snow, which contains large numbers of aggregated microbes (Michaels and Silver, 1988).

1.4.2 Phytoplankton

The photosynthetic component of plankton is termed phytoplankton. The eukaryotic component of phytoplankton, or "microalgae", comprises such ecologically important groups as diatoms, coccolithophores and dinoflagellates; members of the latter group forming the toxic blooms known as red tides. Some of these eukaryotes are small enough to be considered nanoplankton, while other photosynthetic eukaryotes are even small enough to be considered picoplankton, although most picoplankton members are prokaryotes. The prokaryotic component of phytoplankton comprises such cyanobacterial genera as *Synechococcus*, *Prochlorococcus* and *Trichodesmium*. Marine phytoplankton, at the base of the oceanic food chain and responsible for almost half of the global primary productivity, are therefore an ecologically very important group of organisms.

Despite the relative uniformity of the oceans, considerable variations of abundance and diversity within the marine microbial community occur, both spatially and temporally. In oligotrophic waters picophytoplankton, rather than eukaryotic phytoplankton, tend to dominate, and are responsible for most of the primary production (Li *et al.*, 1983). Picophytoplankton have a relative advantage in taking up nutrients, resulting from their small size and consequently higher surface-to-volume ratio. The larger, eukaryotic phytoplankton are therefore out-competed unless the nutrient concentrations are greater than those required for picoplankton (see review by Fogg, 1995). Blooms of a single organism, notably phytoplankton, may arise and dominate the community, when their growth rate exceeds the rate at which they are grazed, or the growth rate of their grazer (*e.g.* Brussaard *et al.*, 1996a).

1.5 Cyanobacteria

Cyanobacteria are Gram negative bacteria which perform plant-like oxygenic photosynthesis with the aid of the light-harvesting pigments chlorophyll *a* and

phycobiliproteins such as phycoerythrin, phycocyanin and allophycocyanin (for review see Carr and Whitton, 1982). The main mode of carbon nutrition is by photoautotrophy. However, many are capable of a degree of photoheterotrophy (Rippka *et al.*, 1979). All cyanobacteria can use ammonium and nitrate for growth (Stanier and Cohen-Bazire, 1977; Gibson, 1984), and many are able to fix nitrogen to ammonium. Many nitrogen-fixers are filamentous, and use heterocysts (specialised cells) for nitrogen-fixation, *e.g.* *Richelia intracellularis*. Others, *e.g.* *Trichodesmium* sp., form dense colonies, the centre of which is anaerobic for nitrogen-fixation, although they can also fix nitrogen in cells which are simultaneously photosynthesising (for review see Capone *et al.*, 1997).

Many cyanobacteria are free-living. However, there are also many examples of symbioses between cyanobacteria and eukaryotes, in which the cyanobacteria receive a safe and prominent place for photosynthesis, and provide the host with fixed carbon or nitrogen (see review by Fogg *et al.*, 1973). Some cyanobacteria are extremophiles, living in such habitats as hot springs, deserts, hypersaline lagoons and permanent ice (see Carr and Whitton, 1982). Many cyanobacteria inhabit freshwater, and some of these may form blooms in eutrophic lakes, producing extremely potent toxins, which can be a serious hazard for metazoan wildlife. The collapse and degradation of such blooms starves other wildlife of oxygen, resulting in considerable environmental problems (for review see Gibson and Smith, 1982).

Cyanobacteria which inhabit the marine environment may be either benthic or planktonic. Benthic forms grow in light intensities of *ca.* 1-2 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Fogg *et al.*, 1973), whilst planktonic forms growing near the surface endure summer midday intensities of 2000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Joint, 1990). Planktonic cyanobacteria are the most diverse and widespread of the marine picophytoplankton, with the greatest contribution to primary production in oligotrophic systems, especially near the bottom of the euphotic zone (Liu *et al.*, 1995).

The four main genera of marine cyanobacteria are *Trichodesmium*, *Synechococcus*, *Prochlorococcus* and *Richelia*. Members of the genus *Richelia* live symbiotically

inside diatoms (see review by Carr and Mann, 1994). *Trichodesmium* spp. are relatively large, ca. 6-10 μm cell diameter, form dense colonies of long filaments, ca. 2 mm diameter, and are globally significant for nitrogen fixation (for review see Capone *et al.*, 1997).

Marine *Synechococcus* and *Prochlorococcus* are very similar genera, both morphologically and genetically, as revealed by 16S rRNA and RNA polymerase analyses (Palenik and Haselkorn, 1992; Urbach *et al.*, 1992). These cyanobacteria are small (0.6-1.6 μm and 0.6-0.8 μm respectively), unicellular, relatively fast-growing and very abundant in the marine environment (10^3 - 10^5 ml⁻¹ and ca. 10^5 ml⁻¹ in surface waters, respectively; e.g. Liu *et al.*, 1995). *Prochlorococcus* spp. were only discovered relatively recently (Chisholm *et al.*, 1988), and differ from *Synechococcus* spp. in possessing the light-harvesting pigments divinyl chlorophylls *a* and *b*. Previously, no *Prochlorococcus* sp. strain had been described which possessed phycobiliproteins. However, recently genes were discovered in a *Prochlorococcus* sp. strain which encoded a phycobiliprotein similar to phycoerythrin found in *Synechococcus* spp. (Hess *et al.*, 1996). Hence, the two genera are even more similar than previously thought. It was once thought that *Synechococcus* was perhaps the most ecologically important genus (Waterbury *et al.*, 1986), however, members of *Prochlorococcus* are now known to have a considerable contribution to phytoplankton biomass and primary production (Goericke and Welschmeyer, 1993).

1.6 Marine *Synechococcus*

1.6.1 The organism

The first reports of truly marine *Synechococcus* spp. were not until 1979 (e.g. Waterbury *et al.*, 1979), when they were discovered in the open ocean by epifluorescence microscopy, fluorescing bright orange when excited with light of 540 nm wavelength, as a result of their phycoerythrin content. The characteristic orange

fluorescence was distinct from the red fluorescence resulting from chlorophyll *a* in eukaryotic phytoplankton. At that time *Synechococcus* was a broad genus encompassing all small unicellular cyanobacteria with ovoid to cylindrical cells, which reproduce by binary fission in a single plane, and lack sheaths (Rippka *et al.*, 1979). However, morphologically similar strains showed considerable genetic heterogeneity, manifested in their vastly different DNA base compositions, which ranged from 39-71 mol % G+C. The "genus" *Synechococcus* was consequently subdivided into three groups, and the group with 47-56 mol % G+C was named *Synechococcus*. All truly marine *Synechococcus* spp. strains which have been examined form a distinct cluster from freshwater and terrestrial *Synechococcus* spp., ranging from 55-63 mol % G+C (Waterbury *et al.*, 1986).

Marine *Synechococcus* divides into subgroups which are equivalent to genera (Waterbury and Rippka, 1989). One group lacks the light-harvesting pigment phycoerythrin and is halotolerant, not requiring elevated salt concentrations for growth. This group can grow equally well in seawater or freshwater, but has only been found in coastal regions, and not in the open ocean. The primary light-harvesting pigment in this group is phycocyanin, which is a disadvantage in open oceans (Wood, 1985). Members of this group were assigned to *Synechococcus* Marine-Cluster C (MC-C) (Waterbury and Rippka, 1989). The other group possesses phycoerythrin as its primary light-harvesting pigment. Members of this group have an obligate requirement for elevated salt concentrations for growth, and are abundant in the euphotic zone of both coastal and open ocean regions. Hence, members of this group, assigned to MC-A (Waterbury and Rippka, 1989), are truly marine (for review see Waterbury *et al.*, 1986). This phycoerythrin-rich group of marine *Synechococcus* also has two distinct subgroups, based on the predominant chromophore associated with phycoerythrin. One group is phycourobilin-rich, and is characteristic of the open oceans. The other has a lower phycourobilin content, and is associated more with shelf waters (Olson *et al.*, 1990).

Molecular analysis of *Synechococcus* spp. strains, by restriction fragment length polymorphism (RFLP) analysis, distinguished marine *Synechococcus* spp. from other

freshwater *Synechococcus* spp. (Douglas and Carr, 1988). However, considerable genetic differences were also found within the marine *Synechococcus* group, using RFLP analysis, with differences between marine strains as great as between marine and freshwater strains (Wood and Townsend, 1990). These data coincide with the apparent genetic diversity revealed earlier by the range of DNA base ratios, 55-63 mol % G+C (Waterbury *et al.*, 1986).

Marine *Synechococcus* spp. cells are mainly coccoid in the natural environment, 0.6-1.6 μm in diameter (see review by Waterbury *et al.*, 1986). Their size tends to increase with depth in the euphotic zone (*e.g.* Burkill *et al.*, 1993). In laboratory culture the cells become rod-shaped, up to 0.6 x 1.6 μm ; however, if grown in a light-dark cycle the cells remain coccoid (Waterbury *et al.*, 1986). The Gram negative cell wall is made of a peptidoglycan layer surrounded by an outer membrane. Some strains, such as *Synechococcus* sp. WH7803, have a highly structured outer envelope, while others have external projections (spinae) (see review by Waterbury *et al.*, 1986). *Synechococcus* spp. have characteristic location and spacing of the photosynthetic thylakoids. They are located peripherally, just inside the cytoplasmic membrane, and separated by 40-50 nm, which is just sufficient to house the light-harvesting phycobilisomes, which are situated on the outer surface of the thylakoid membranes (see review by Waterbury *et al.*, 1986).

Like most other cyanobacteria, the primary photosynthetic pigment in *Synechococcus* spp. is chlorophyll *a*, with phycobiliproteins as accessory light-harvesting pigments (Waterbury *et al.*, 1979). Most cyanobacteria possess the phycobiliprotein phycocyanin, and truly marine *Synechococcus* spp. strains also possess phycoerythrin. Phycocyanin absorbs light from 550-645 nm, while phycoerythrin absorbs 490-560 nm. The relative proportions of phycocyanin and phycoerythrin are responsible for the colour of the *Synechococcus* sp. strain (see review by Waterbury *et al.*, 1986). If the strain lacks phycoerythrin then it appears blue-green. Increasing amounts of phycoerythrin give the strain an olive-green to reddish-orange colour. All truly marine strains, inhabiting the open ocean, are reddish-orange (phycoerythrin-rich), able to absorb shorter wavelengths, nearer to the wavelength which best

penetrates seawater (450 nm). Hence, they are able to photosynthesise at greater depths than phycocyanin alone would allow.

Since marine *Synechococcus* spp. have large amounts of phycoerythrin, as well as phycocyanin and allophycocyanin, they are able to photosynthesise at maximum rates in low light intensities (see review by Waterbury *et al.*, 1986). Hence, marine *Synechococcus* spp. thrive in conditions of low light, such as $10 \mu\text{E m}^{-2} \text{s}^{-1}$, further down the water column, but are able to decrease their phycoerythrin content up to 20-fold with an increase in light intensity. Therefore, they can tolerate light in surface waters at $2000 \mu\text{E m}^{-2} \text{s}^{-1}$ without photoinhibition (Kana and Glibert, 1987). The optimum growth temperature for most *Synechococcus* spp. strains in culture is 20-25°C. Growth is severely limited below 6°C and above 30°C (Waterbury *et al.*, 1986).

Like many other cyanobacteria, all marine *Synechococcus* spp. strains appear to be obligate photoautotrophs, yet may be able to assimilate some organic compounds, although they cannot use these as their sole carbon source. All strains appear able to utilise nitrate and ammonium as a sole nitrogen source, and some can utilise urea (see review by Waterbury *et al.*, 1986). Marine *Synechococcus* spp. can grow at high rates under poor nutrient conditions. This gives them a considerable advantage in oligotrophic waters in competing with other phytoplankton which need higher nutrient concentrations to grow effectively (Kudoh *et al.*, 1990).

1.6.2 Distribution and abundance

Marine *Synechococcus* spp. have commonly been found in the euphotic zone throughout the world's oceans at concentrations of 10^3 - 10^5 cells ml^{-1} , but can range from a few to 10^6 ml^{-1} (Olson *et al.*, 1990; Burkill *et al.*, 1993; Liu *et al.*, 1995). They have even been observed in polar waters at low concentrations (*ca.* 1 ml^{-1} ; Robineau *et al.*, 1994).

The vertical distribution of *Synechococcus* spp. through the water column is highly dependent upon the penetration of photosynthetically active radiation (PAR). The lower limit for *Synechococcus* spp. growth is approximately at the depth to which PAR penetrates with 1% transmittance. In clear, oligotrophic waters this may be as much as 150 m (Iturriaga and Marra, 1988), but it may be considerably less in more nutrient-rich areas, especially if concentrations of eukaryotic algae nearer the surface are high. *Synechococcus* spp. cells may be found at greater depths as a result of mixing, and their depth profile may be greatly affected by the presence of a shallow thermocline, trapping many cells in the surface mixed layer (Iturriaga and Marra, 1988). It appears that maximum *Synechococcus* spp. concentrations are often at, or near, the surface where the water column is weakly stratified (Jochem, 1995). However, where the water column is strongly stratified there is often a distinct subsurface maximum just below the surface mixed layer, if PAR is >1% (e.g. Burkill *et al.*, 1993). Subsurface *Synechococcus* spp. maxima are also often observed just above the chlorophyll maximum (Burkill *et al.*, 1993; Jochem, 1995).

Observations of *Synechococcus* spp. abundance in the Indian Ocean have shown greater *Synechococcus* spp. concentrations in more oligotrophic waters (Burkill *et al.*, 1993). However, *Synechococcus* spp. concentrations generally appear to be greatest in coastal waters rather than in the open ocean (Glover, 1985; Olson *et al.*, 1990; Jochem, 1995).

Synechococcus spp. abundance varies temporally, ranging from annual to diurnal cycles. *Synechococcus* spp. concentrations in temperate waters, especially in coastal regions, are strongly influenced by seasonal changes in water temperature (Waterbury *et al.*, 1986). The annual cycle (Fig. 1.4) has been well studied in Woods Hole Harbour, NW Atlantic (Waterbury *et al.*, 1986). Annual cycles in these temperate waters are usually characterised by a relatively low concentration throughout winter, which seeds a spring bloom at the beginning of April. Water temperature of 6°C signals the onset of the spring bloom and the decline in winter (Fig. 1.4).

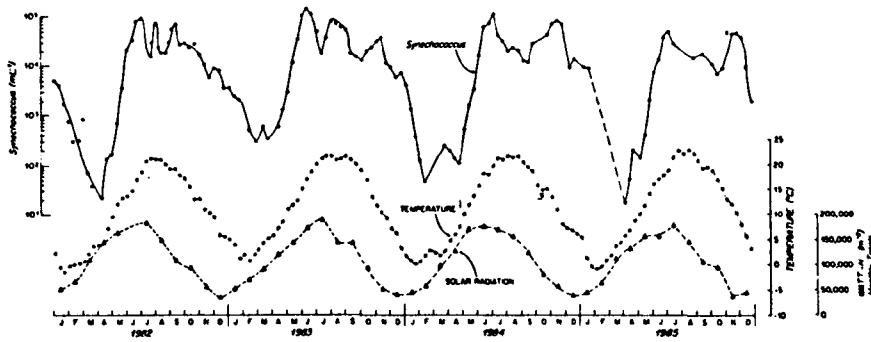


Figure 1.4 Annual cycles of *Synechococcus* spp. abundance in Woods Hole harbour, 1982-1985. *Synechococcus* spp. abundance, determined from surface samples, is compared with temperature and monthly totals of solar radiation. Throughout much of each annual cycle the individual points are weekly averages from several counts. Reproduced from Waterbury *et al.*, 1986.

Synechococcus spp. concentrations may vary greatly over a few days, resulting from short-term weather patterns. Storms and rainfall can result in increased growth by adding nutrients to the euphotic zone, and cloud cover may decrease growth (see review by Waterbury *et al.*, 1986). Storms disrupt the surface mixed layer and bring up nutrients by mixing deeper water. Diurnal cycles of *Synechococcus* spp. have also been observed *in situ*. However, differences in the cycles occur depending on the oceanographic region. In coastal regions *Synechococcus* spp. cells divide at midday (Carpenter and Campbell, 1988), whereas in oceanic regions cell division occurs at dusk (Kudoh *et al.*, 1990).

1.6.3 Ecological importance

Considerable interest has been placed in marine *Synechococcus* spp. because of their widespread abundance, yet also because of early reports of their sizeable contribution to oceanic, and therefore global, primary productivity (see below). *Synechococcus* spp. production is greatest near the surface, and decreases with depth (*e.g.* Liu *et al.*, 1995). However, the relative contribution of *Synechococcus* spp. to total production often increases towards the bottom of the euphotic zone (Iturriaga and Marra, 1988; Liu *et al.*, 1995). Differences in the relative contribution of *Synechococcus* spp. to

primary production usually reflect the relative success of eukaryotic phytoplankton rather than *Synechococcus* spp., at a particular site and time (see review by Waterbury *et al.*, 1986).

Earlier studies reported that *Synechococcus* spp. contributed up to 95% of total primary production near the bottom of the euphotic zone (Iturriaga and Mitchell, 1986; Iturriaga and Marra, 1988). However, when production was averaged throughout the euphotic zone, estimates suggested *Synechococcus* spp. contributions of 10-25% of total production in the Sargasso Sea, and 5-10% in coastal waters near Woods Hole (Waterbury *et al.*, 1986). However, these values were confounded by *Prochlorococcus* spp., which were discovered more recently (Chisholm *et al.*, 1988). *Prochlorococcus* spp. overlap with *Synechococcus* spp. in size range and distribution in the water column (*e.g.* Landry *et al.*, 1996). Hence, the precise contribution of *Synechococcus* spp. alone cannot be accurately assessed with traditional size-fractionated productivity experiments. However, estimates of production can be made with the aid of flow cytometry or epifluorescence microscopy to distinguish *Synechococcus* spp. from *Prochlorococcus* spp. (Burkill *et al.*, 1993; Liu *et al.*, 1995). Such estimates have suggested that *Synechococcus* spp. may contribute much less than *Prochlorococcus* spp. to total primary production, but that they may still contribute *ca.* 5% of total production in the open ocean (Liu *et al.*, 1995).

The final fate of carbon fixed by *Synechococcus* spp. is uncertain. However, these cyanobacteria are grazed by a range of small microbes, especially heterotrophic nanoflagellates (HNFs) and ciliates (*e.g.* Kudoh *et al.*, 1990). Kudoh *et al.* (1990) observed that HNFs were responsible for one third of the loss of *Synechococcus* spp. from grazing, while ciliates and larger, rarer micrograzers were responsible for the remaining two thirds. High *Synechococcus* spp. growth rates have been observed (up to 1.0 day⁻¹; Burkill *et al.*, 1993; Liu *et al.*, 1995). However, correspondingly high mortality rates from grazing by microzooplankton have also been observed (up to 1.2 day⁻¹; Burkill *et al.*, 1993; Liu *et al.*, 1995), suggesting a tight coupling between production and grazing.

1.7 Molecular Techniques

Traditional methods for analysing specific viruses and virus populations have concentrated on conventional microbiological techniques, such as plaque assay and most probable number assay (*e.g.* Suttle and Chan, 1993; Waterbury and Valois, 1993), and transmission electron microscopy (TEM) (*e.g.* Frank and Moebus, 1987; Borsheim *et al.*, 1990). However, these methods are laborious and time-consuming. TEM lacks specificity, and can give little information about the virus-host systems represented. Furthermore, culture techniques are limited to the subset of viruses which infect the specific host strains possessed in the laboratory, and therefore may not detect a considerable proportion of the species-specific viruses in the environment.

New techniques have recently been developed in order to overcome some of the above problems. Steward *et al.* (1992a; 1992b) used radiotracers to analyse virus production rates via the incorporation of ^{32}P -orthophosphate and ^3H -thymidine into virus particles; a technique which has since been used successfully in other studies (Fuhrman and Noble, 1995; Steward *et al.*, 1996). Brussaard *et al.* (1995) estimated marine virus production indirectly by dissolved esterase activity, produced from lysis of phytoplankton. Hennes and Suttle (1995) used novel, highly fluorescent nucleic acid dyes (Hirons *et al.*, 1994) to enumerate marine virus populations by epifluorescence microscopy. The same group of dyes has been used to enumerate marine bacterial populations by flow cytometry (Marie *et al.*, 1996), and it may be possible to apply this to rapid enumeration of marine virus populations. However, each of these techniques also lacks specificity, and cannot distinguish natural individual virus groups; although Hennes *et al.* (1995) used fluorescent-stained cultured viruses to examine *in situ* populations of bacteria and cyanobacteria.

Wilson (1994) attempted the development of an immuno-assay to enumerate cyanophages in the marine environment, by raising antibodies to a cyanophage. However, the attempt was unsuccessful because the sensitivity of cyanophage

detection was insufficient for environmental application, and non-specific binding of the antibody resulted in false positives (Wilson, 1994).

Currently, the most promising techniques for analysing specific virus populations in the marine environment are molecular techniques (for review see Wilson and Mann, 1997). Initial attempts used restriction analysis of virus DNA to investigate genetic variation (Cottrell and Suttle, 1991; Wilson *et al.*, 1993) and DNA sequences as gene probes, by Southern hybridisation (*e.g.* Ogunseitan *et al.*, 1992; Cottrell and Suttle, 1995b; for reviews see Gerba *et al.*, 1989; Lopez-Pila *et al.*, 1993). RFLP analysis has often been used for rapid investigation of genetic diversity amongst similar organisms, such as *Synechococcus* spp. (*e.g.* Douglas and Carr, 1988; Wood and Townsend, 1990; Ernst *et al.*, 1995).

Hybridisation experiments have been used to estimate the genetic similarity of marine vibriophages (Kellogg *et al.*, 1995) and marine algal viruses (Cottrell and Suttle, 1995b). Ogunseitan *et al.* (1992) used radiolabelled phage DNA probes to determine the distribution of *Pseudomonas aeruginosa* phages in environmental samples, but could only detect down to 10^3 - 10^4 PFU. Hence, Southern hybridisation lacks sufficient sensitivity when applied to environmental samples (Ogunseitan *et al.*, 1992; for review see Lopez-Pila *et al.*, 1993), and has several other practical disadvantages (for review see Sobsey, 1993).

1.7.1 Polymerase chain reaction

Most techniques now are based on the more sensitive and more rapid polymerase chain reaction (PCR) (*e.g.* Graff *et al.*, 1993; Chen and Suttle, 1995b; Straub *et al.*, 1995; for review of PCR see Giovannoni, 1991; Zimmerman and Mannhalter, 1996). In contrast to direct Southern hybridisation, detecting 10^2 - 10^3 infectious units, PCR has been used to detect hepatitis A viruses, enteroviruses and rotaviruses at 0.05-0.3 PFU, or 1-100 virus particles (for review see Sobsey, 1993). PCR followed by

hybridisation has been shown to be 10^5 - 10^7 times more sensitive than direct hybridisation in detecting enteroviruses from water (Kopecka *et al.*, 1993). PCR can also be made more sensitive with the use of a second pair of primers, internal to the first set (nested), or a single internal primer used with one of the original primers (semi-nested), for a second round of amplification (*e.g.* Romanowski *et al.*, 1993; Puig *et al.*, 1994; Chen and Suttle, 1995b; Zhang and Martineau, 1996). This confirms the presence of a correct PCR product from the first round of amplification, but can also improve the specificity as well as the sensitivity of the primers (Chen and Suttle, 1995b).

Complete analysis of PCR products can be performed by obtaining sequence data (*e.g.* Chen *et al.*, 1996). PCR, followed by sequence analysis, has permitted the recent phylogenetic analysis of specific marine virus populations (Chen and Suttle, 1995a; 1996; Chen *et al.*, 1996). However, this is relatively time-consuming. A more rapid and accurate representation of the clonal composition of a mixed population may be obtained by denaturing gradient gel electrophoresis (DGGE). This technique separates PCR products of the same size but of different base composition, on the basis of the melting temperature of the DNA, as described by Muyzer *et al.* (1993). DGGE has since been increasingly used to analyse mixed microbial populations in the natural environment (Muyzer *et al.*, 1993; Wawer and Muyzer, 1995; Teske *et al.*, 1996). An alternative method to DGGE is the recent application of bisbenzimidide-PEG to the separation of PCR products (Wawer *et al.*, 1995; Kristensen and Borresen-Dale, 1997). Similarly, this separates PCR products on the basis of base composition, but is a more rapid and technically easier method than DGGE. Another method used for analysis of PCR products is RFLP, though the data obtained are rather limited. However, RFLP has been used for very rapid analysis of viral PCR products (Chen *et al.*, 1996; Jackson *et al.*, 1996; Chang *et al.*, 1997; Dubois *et al.*, 1997).

PCR of virus DNA can be facilitated by purification and concentration of the viruses by immunoaffinity capture (Graff *et al.*, 1993; for review see Sobsey, 1993). However, this would require either a single common antigen or a battery of antisera

to detect all the desired viruses from the marine environment (see review by Sobsey, 1993). Any methods of concentration or purification of viruses will likely reduce the accuracy of quantification of viruses.

1.7.2 PCR analysis of marine viruses

Commonly, analysis of genetic diversity of microbes has exploited the highly conserved ribosomal RNA (rRNA) gene (for review see Giovannoni *et al.*, 1995). However, since viruses do not contain rRNA the conserved DNA polymerase gene has been targeted (Chen and Suttle, 1995a; 1995b), which is present in all DNA viruses. Hence, genetic analysis of marine algal virus populations has been possible (Chen and Suttle, 1996; Chen *et al.*, 1996).

Chen *et al.* (1994) designed highly degenerate PCR primers, based on the DNA polymerase gene from three microalgal viruses, to amplify specifically algal-virus DNA. Chen and Suttle (1995a) used the same primers to amplify DNA from viruses infecting three genera of microalgae: *Chlorophyceae*; *Prasinophyceae*; and *Prymnesiophyceae*, although the primers would not amplify DNA from viruses of marine brown algae (*Phaeophyceae*). Using the same primers, Chen and Suttle (1996) constructed a dendrogram based on sequence analysis of the amplified fragments from 13 cultured virus clones infecting the above three microalgal genera. They observed that microalgal viruses formed a distinct group, Phycodnaviridae, more similar to each other than to other viruses of eukaryotes (Chen and Suttle, 1996). These algal-virus specific primers were then used to amplify DNA which had been concentrated from natural seawater samples in the Gulf of Mexico (Chen *et al.*, 1996). PCR products from a single station were cloned and examined for genetic diversity by RFLP and sequence analysis (Chen *et al.*, 1996). The results were similar to those previously obtained by DNA hybridisation studies (Cottrell and Suttle, 1995), demonstrating that genetic diversity amongst genotypes within a

sample was as great as that between samples from different oceans (Chen *et al.*, 1996).

An alternative approach to using the DNA polymerase gene is to identify a gene encoding a conserved capsid protein. This has been demonstrated with viruses infecting marine brown algae and viruses infecting several different species of fish. A coat protein gene was identified in *EsV*, a virus infecting the brown alga *Ectocarpus siliculosus* (Phaeophyceae) (Klein *et al.*, 1995). PCR primers were designed based on conserved regions of this gene (Brautigam *et al.*, 1995), and used to detect *EsV* DNA in extracts of unialgal *Ectocarpus* cultures from the coasts and oceans around the world, discovering that at least 50% contained viral DNA (Sengco *et al.*, 1996). Mao *et al.* (1997) used PCR primers based on conserved regions in the major capsid protein of fish iridoviruses, and observed genetic diversity among nine isolates, according to the PCR products. Similarly, Nishizawa *et al.* (1997) obtained sequence data of PCR products from a coat protein gene of fish nodaviruses, and subsequently constructed a dendrogram.

Such molecular studies have shown that marine viruses are genetically very diverse and widely distributed (Cottrell and Suttle, 1995b; Chen *et al.*, 1996). Molecular techniques, in particular PCR, should therefore be very useful for studying specific virus populations and their impact upon their hosts. Whilst it is well established that molecular techniques can detect uncultivable organisms (*e.g.* Giovannoni *et al.*, 1990a; Fuhrman *et al.*, 1993a; 1994), to date there has been a dearth of data on the specificity range of molecular techniques applied to marine viruses (Chen and Suttle, 1995a). However, in theory, such techniques may be able to detect all viruses infecting a specific host species, regardless of the host strain. Together with the inherent sensitivity of PCR to minute quantities of DNA and the rapidity of molecular techniques, these characteristics make molecular techniques considerably more useful tools than those used traditionally. The one big disadvantage, however, with such techniques is the current inability to distinguish between infective and noninfective viruses.

1.7.3 Competitive PCR

Whilst machines are now available for quantifying PCR products (from Perkin-Elmer), they are prohibitively expensive for routine use. Such machines are based on the detection of fluorescently labelled products in the early cycles when PCR fidelity is highest. However, PCR may be used as a quantitative assay with the use of an internal standard (for reviews see Diviacco *et al.*, 1992; Siebert and Larrick, 1992; Zimmerman and Mannhalter, 1996). The exponential nature of the PCR means that any inhibiting contaminant or small variations in the initial amplification steps will result in considerable inaccuracies with the amount of product (see review by Diviacco *et al.*, 1992). Hence, if an internal standard is added to the PCR then both the target and the standard DNAs should be affected equally (see review by Diviacco *et al.*, 1992). Since different pairs of primers may have different amplification efficiencies, it is most appropriate to design a competitor piece of DNA which has the same primer binding sites as the target DNA, and hence competes with the target (Gilliland *et al.*, 1990). The ratio of the target to competitor products should accurately reflect the initial ratio (Diviacco *et al.*, 1992), and hence, using a known amount of competitor, the initial target concentration can be evaluated. Whilst PCR has been reported to be quantitative without an internal standard, the range of quantification was very small (Romanowski *et al.*, 1993). Competitive PCR (cPCR) still remains a far more reliable method (see review by Zimmerman and Mannhalter, 1996), occasionally allowing quantification of the target over a range of six orders of magnitude (Payan *et al.*, 1997). Quantification has also been shown to be possible by cPCR using nested and semi-nested primers (*e.g.* Cammarota *et al.*, 1996).

The products of the competitor and target can be distinguished most easily by different lengths (by addition or deletion of short sequences in the competitor; *e.g.* Diviacco *et al.*, 1992), or by a mutation in the competitor creating a novel restriction site (*e.g.* Gilliland *et al.*, 1990). Products are separated by conventional gel electrophoresis and can be easily quantified by densitometric scanning of a Polaroid image of a gel stained with ethidium bromide or an alternative fluorescent dye (*e.g.*

Diviacco *et al.*, 1992). Alternatively, the product can be labelled with radioactive or fluorescent dNTPs (*e.g.* Gilliland *et al.*, 1990), and the product excised from the gel and quantified by liquid scintillation or direct autoradiography and scanning of a dried gel (for review see Zimmerman and Mannhalter, 1996). Another, increasingly used technique is to label the product with a fluorescent primer, then quantify it using an automated DNA sequencer (Cammarota *et al.*, 1996; Thiery *et al.*, 1996; Hammerle *et al.*, 1997).

A standard method of accurate quantification by cPCR, based on the construction of a calibration curve, was described by Zachar *et al.* (1993). Most cPCR methods run the PCR beyond the exponential phase, into the plateau phase (see review by Zimmerman and Mannhalter, 1996). For accurate quantification the amplification efficiencies of the target and competitor must therefore be equal. However, Zachar *et al.* (1993) maintain that if the PCR is restricted to the exponential phase then quantification is valid even when the efficiencies are different, providing that the ratio of the efficiencies remains constant. Hence, it would not be necessary to prove the equality of the two amplification efficiencies (Zachar *et al.*, 1993). Lee *et al.* (1996) have since used this method successfully to enumerate an uncultured bacterial strain in soil.

Competitive PCR has been used to quantify individual strains of bacteria and cyanobacteria in environmental samples (Leser, 1995; Leser *et al.*, 1995; Lee *et al.*, 1996; Moller and Jansson, 1997), and individual strains of viruses in clinical samples (Clark *et al.*, 1996; Hammerle *et al.*, 1996; Thiery *et al.*, 1996; Hammerle *et al.*, 1997; Payan *et al.*, 1997). However, to date cPCR has not yet been applied to quantification of complex, diverse assemblages of either bacteria or viruses.

1.8 Aims and Outline to the Research

The main aim of this study was to develop molecular probes which would specifically detect marine cyanophages, then to apply these probes to interrogate natural cyanophage populations.

In particular, interest was placed in cyanophages which infect marine *Synechococcus* spp.. At the onset of this study cyanophages were known to be widely distributed throughout the world's oceans, and were thought to play an important role in the mortality and clonal composition of *Synechococcus* spp. (Proctor and Fuhrman, 1990; Suttle and Chan, 1993; Waterbury and Valois, 1993; Wilson *et al.*, 1993; Suttle and Chan, 1994; Wilson, 1994). Molecular techniques were in use for the molecular analysis of marine systems, although these had hardly been applied to marine cyanophages (Wilson, 1994). Hence, molecular tools were developed, continuing work begun by William Wilson in this laboratory, in order to gain a deeper insight into these ecologically important populations.

In order to obtain controls for the development of cyanophage-specific probes, attempts were made to isolate cyanophages from different oceanographic provinces. Preliminary characterisation, both morphological and molecular, was made of those cyanophages which were isolated. Novel marine bacteria were also isolated, and attempts were made to isolate phages for such, as additional controls for the probes. Chapter three deals with the isolation and characterisation of these viruses.

William Wilson had previously cloned a DNA fragment from each of three marine cyanophages, which showed limited homology to each other by Southern hybridisation, and had obtained a very limited amount of sequence data from two of these fragments, which revealed limited homology to gene 20 from the coliphage T4 (Wilson, 1994). In Chapter four, additional DNA fragments were isolated, to obtain the entire gene homologue in each of the three cyanophages. The open reading frames were subsequently sequenced on both strands, to complete the sequencing

begun by Wilson. A DNA alignment was constructed, of the three cyanophage sequences together with the T4 sequence, and a pair of PCR primers was designed from conserved regions, in order to amplify specifically cyanophage DNA.

Chapter five deals with the screening of the PCR primers designed in Chapter four for their suitability as cyanophage-specific probes, so that they might later be applied to the natural environment. After the PCR had been optimised, the specificity and sensitivity of the primers were determined, together with the readiness with which they could be applied to marine samples. A substantial avenue to this research which developed was the possibility of developing a molecular technique for the rapid and comprehensive quantification of marine cyanophages *in situ*. Hence, an attempt was made to develop a suitable quantitative competitive PCR, based on the strategy described by Zachar *et al.* (1993). In order to develop a technique for the molecular characterisation of marine cyanophage populations, attempts were made to characterise the PCR products obtained from cultured cyanophages.

Finally, in Chapter six the cyanophage-specific PCR primers were applied to seawater samples which were collected on an Atlantic cruise, in order to gain molecular information from natural cyanophage populations. PCR products were cloned and sequenced, and the resulting data were analysed.

Chapter 2:

Materials and Methods

2.1 Strains and Plasmids

Table 2.1 Cyanobacterial strains. All *Synechococcus* sp. strains were donated by John Waterbury, Woods Hole Oceanographic Institute, USA (Waterbury *et al.*, 1986), and belong to Marine Cluster A. *Prochlorococcus* sp. strains were obtained from David Scanlan, Warwick (see Scanlan *et al.*, 1996). For modified K/10 medium see Scanlan *et al.* (1996).

Strain	Origin	Growth Media
<i>Synechococcus</i> sp. WH7803	North Atlantic (33° 44.8' N, 67° 29.8' W)	ASW
<i>Synechococcus</i> sp. WH8018	Woods Hole, USA	ASW
<i>Synechococcus</i> sp. WH8103	Sargasso Sea (28° 30' N, 67° 23.5' W)	ASW
<i>Prochlorococcus</i> sp. MED	Mediterranean Sea (43° 12.15' N, 6° 52' E)	Modified K/10
<i>Prochlorococcus</i> sp. SARG	Sargasso Sea (28° 59' N, 64° 21.5' W)	Modified K/10

The main strain of *Synechococcus* sp. used throughout this study was WH7803 "Warwick" (formerly designated DC2).

The strain of *Escherichia coli* used throughout this study was TG1, genotype: K12, (*lac-pro*), *supE*, *thi*, *hsdD5*, *F'traD36*, *proA⁺B⁺*, *lacIq*, *lacZ*(M15) (Carter *et al.*, 1985).

Table 2.2 Plasmids. pB063₇ and all pWHW and pNJF plasmids are based on pUC19. References: (1) Yanisch-Perron *et al.* (1985); (2) Wilson (unpublished results); (3) Wilson (1994); (4) Chapter 4 this study; (5) Chapter 5 this study.

Plasmid	Relevant characteristics	Reference
pUC19	Ap ^R , <i>lacZ</i> .	1
pB063 ₇	1.8 kb <i>Bam</i> HI/ <i>Pst</i> I insert cloned from cyanophage strain S-BnM1	2
pWHW01	3.5 kb <i>Bam</i> HI insert cloned from cyanophage strain S-WHM1.	3
pWHW02	1 kb <i>Bam</i> HI/ <i>Eco</i> RI insert sub-cloned from pWHW03 insert (cyanophage strain S-PM2).	3
pWHW03	20 kb <i>Bam</i> HI insert cloned from cyanophage strain S-PM2.	3
pWHW04	1.5 kb <i>Pst</i> I/ <i>Eco</i> RI insert cloned from cyanophage strain S-BnM1.	3
pNJF1	2 kb and 1 kb <i>Xba</i> I inserts cloned from cyanophage strain S-PM2.	4
pNJF2	2 kb <i>Xba</i> I insert sub-cloned from pNJF1 inserts (cyanophage strain S-PM2).	4
pNJF3	193 bp <i>Sph</i> I/ <i>Pvu</i> II insert cloned from pUC19 into <i>Eco</i> NI site in pWHW04.	5
pNJF4	3 kb <i>Eco</i> RV insert cloned from cyanophage strain S-WHM1.	4
pNJF5	5.1 kb <i>Eco</i> RI insert cloned from cyanophage strain S-BnM1.	4

Table 2.3 Virus strains. Family: M - Myoviridae; P - Podoviridae; S - Siphoviridae; Ph - Phycodnaviridae. Host: WH7803, WH8018, WH8103, WH8012, SYN48 and SNC2 are all "red" strains of *Synechococcus* sp. and SNC1 is a "green" strain of *Synechococcus* sp.. Those host strains in parentheses are not infected by the virus strain. H2, H4 and H54 are all heterotrophic bacteria. References: (1) Wilson (1994); (2) Waterbury and Valois (1993); (3) Chapter 3 this study; (4) Suttle and Chan (1993); (5) Hu *et al.* (1981); (6) Suttle and Chen (1992); (7) Frank and Moebus (1987); (8) Jacobsen *et al.* (1996); (9) Keizo Nagasaki (personal communication); (10) Cottrell and Suttle (1991); (11) Demerec and Fano (1945).

Strain	Family	Host	Place of isolation	Ref.
S-PM2	M	WH7803, WH8012, WH8018. (WH8103)	Plymouth Sound, UK	1
S-BnM1	M	WH7803. (WH8018)	Bergen, Norway	1
S-WHM1	M	WH7803, WH8012. (WH8018, WH8103)	Woods Hole, USA	1
S-WHM2	M	WH7803, WH8012	Woods Hole, USA	2
S-RSM1	M	WH7803	Red Sea	1
S-RSM2	M	WH7803	Red Sea	1
S-BM1	M	WH7803, WH8012, WH8018. (WH8103)	Bermuda	1
S-BM3	M	WH7803, WH8103. (WH8018)	Sargasso Sea	3
S-BM4	M	WH7803, WH8018. (WH8103)	Bermuda	3
S-BM5	M	WH8018. (WH7803)	Bermuda	3
S-BM6	M	WH7803	Sargasso Sea	3
S-BP1	P	WH8018. (WH7803, WH8103)	Bermuda	3
S-BP2	P	WH8018. (WH7803, WH8103)	Bermuda	3
S-BP3	P	WH7803. (WH8018, WH8103)	Bermuda	3
Φ12	P	WH8018. (WH7803, WH8103)	Gulf Stream	2
Φ14	M	WH8103. (WH7803, WH8018)	Gulf Stream	2
S-MM1	M	WH7803	Miami, USA	1
S-MM2	M	WH7803	Miami, USA	1
S-MM3	M	WH7803	Miami, USA	1
S-MM4	M	WH7803	Miami, USA	1
S-MM5	M	WH7803	Miami, USA	1
S-MM7	M	WH7803	Miami, USA	1
Φ2	M	WH7803, WH8018, WH8012. (WH8103)	Sargasso Sea	2
Φ9	M	WH7803, WH8018, WH8103, WH8012	Woods Hole	2
Φ33	M	WH7803	Gulf Stream	2
Φ34	M	WH8103. (WH7803)	Gulf Stream	2
S-PWM1	M	WH7803	Gulf of Mexico	4
S-PWM3	M	WH7803, SYN48, SNC2, SNC1	Gulf of Mexico	4
AN-15	M	<i>Anabaena</i> sp. PCC7120	Freshwater	5
PW3a-P1	M	<i>Vibrio natriegens</i>	Gulf of Mexico	6
H2/1	M	H2	North Sea	7
H4/4	P	H4	North Sea	7
H54/1	S	H54	North Sea	7
PpV-01	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	8
PpV-02	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	9
PpV-04	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	9
PpV-05	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	9
PpV-06	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	9
MpV-Sp1	Ph	<i>Micromonas pusilla</i>	Southern California	10
T4	M	<i>Escherichia coli</i>		11

2.2 Chemicals

All chemicals used were of analytical grade from BDH Chemicals and Sigma Chemicals, unless otherwise stated. Restriction enzymes and their buffers were supplied by GIBCO BRL Life Technologies Ltd., Middlesex, UK. ^{32}P -dCTP and ^{35}S -dATP were supplied by Amersham International plc.. Alkaline phosphatase was supplied by Boehringer Mannheim, Mannheim, Germany. Bacto-tryptone and bacto-agar were supplied by Difco Laboratories Ltd.. Acrylamide and N,N' methylene-bis-acrylamide were supplied by BioRad Laboratories. HA-Yellow was supplied by Hans Analytik, Fahrenheitstr. 1, D-28539 Bremen, Germany. TOTO-1 was supplied by Molecular Probes Europe BV, PoortGebouw, Rijnsburgerweg 10, 2333 AA Leiden, Netherlands.

2.3 Equipment

Gel tanks for DNA gel electrophoresis were supplied by Pharmacia, Bucks. and Flowgen Instruments Ltd., Lynn Lane, Shenstone, Lichfield, Staffs.. Gels were photographed using 665 Polaroid film. The GeneClean II kit was obtained from BIO 101 Inc., 1070 Joshua Way, Vista, CA 92083, USA. The QIAquick gel extraction kit was obtained from QIAGEN Ltd., Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey. The DIG DNA labelling and detection kit was obtained from Boehringer Mannheim. Microtest flexible 96-well assay plates used for preparing DNA sequencing reactions were supplied by Becton Dickinson, Oxnard, USA and the sequencing apparatus was obtained from Cambridge Electrophoresis Ltd., Cambridge, UK. Automated sequencing was performed using an Applied Biosystems 373A DNA Sequencer from Applied Biosystems, Birchwood Science Park North, Warrington, Cheshire. Hybond C nitro-cellulose and Hybond N nylon were supplied by Amersham International plc.. X-ray film (type RX) was obtained from Fuji Film Co., Tokyo, Japan. Photographic paper was obtained from Eastman Kodak Company, Rochester, NY 14650, USA. Cyanophages were visualised and

enumerated at 1000x magnification, under oil, on a Nikon Labophot-2A fluorescence microscope using filter block B-2A (excitation wavelength 450-490 nm; dichromic mirror 510 nm; barrier filter 520 nm). PCR was performed using a Perkin-Elmer DNA thermal cycler from Perkin-Elmer Ltd., Post Office Lane, Beaconsfield, Bucks., and PCR products were quantified using a computing densitometer from Molecular Dynamics Ltd., 4 Chaucer Business Park, Kemsing, Sevenoaks, Kent, and using Image Quant (version 3.3) software. PCR products were cloned using the Original TA Cloning Kit, version E, from Invitrogen BV, De Schelp 12, 9351 NV Leek, Netherlands. 0.2 μm and 2 μm polycarbonate filters were supplied by Poretics Corporation 111 Lindbergh Ave., Livermore, CA 94550-9520, USA. 0.02 μm Anodisc 25 filters and 0.45 μm cellulose nitrate filters were supplied by Whatman Ltd., Maidstone, England. Filtron Mini-Ultrasettes for tangential flow filtration were obtained from Flowgen Instruments Ltd. and the peristaltic pump used was a Verder peristaltic pump 2010 from Flowgen. Other equipment used on the AMT-2 cruise was from Research Vessel Services, Southampton Oceanography Centre, Southampton, UK. Electron microscopy was performed using a Joel JEM-100s transmission electron microscope, Joel Ltd., Tokyo, Japan.

2.3.1 Centrifuges and rotors

Eppendorf tubes used for concentrating small volumes (< 2 ml) of cells and DNA, or for separating aqueous from organic phases were centrifuged in a bench-top MSE microcentaur, MSE Scientific Instruments, Sussex, UK, at 12500 rpm. Glass Universals, holding up to 25 ml, were centrifuged in a bench-top Wifug Labor-50M centrifuge at 4500 rpm.

Larger quantities were centrifuged in an MSE Hi-Spin 21 centrifuge. Volumes up to 30 ml were centrifuged in Oakridge tubes in a JA-20 rotor, while volumes up to 250 ml were centrifuged in 250 ml polycarbonate centrifuge tubes in a JA-10 rotor.

Ultracentrifugation was performed using a Beckman preparative ultracentrifuge, Beckman Instruments Inc., California, USA. Small volumes (14 ml) were centrifuged in 14 ml ultracentrifuge tubes in an SW40Ti rotor, at 30000 rpm.

2.4 Media

Water for all media preparation was obtained from an Elgastat Spectrum system (Elga Ltd., Bucks., UK) containing a reverse osmosis system followed by ion-exchange and carbon filtration. Media were sterilised in gas autoclaves at 115°C, 15 pounds inch⁻² for 20 min for volumes up to 1 l and 30 min for larger volumes. Millipore (0.22 µm) filters, from Millipore (UK) Ltd., Middlesex, were used for filter-sterilising heat-labile solutions.

2.4.1 Medium for growth of *Synechococcus* sp. strains

Table 2.4 ASW medium (Wyman *et al.*, 1985).

ASW	g l ⁻¹	*Trace metal stock	g l ⁻¹
NaCl	25	H ₃ BO ₃	2.86
MgCl ₂ ·6H ₂ O	2	MnCl ₂ ·4H ₂ O	1.81
KCl	0.5	ZnSO ₄ ·7H ₂ O	0.222
NaNO ₃	0.75	Na ₂ MoO ₄ ·2H ₂ O	0.39
MgSO ₄ ·7H ₂ O	3.5	CuSO ₄ ·5H ₂ O	0.008
CaCl ₂ ·2H ₂ O	0.5	Co(NO ₃) ₂ ·6H ₂ O	0.00494
Tris base	1.1	FeCl ₃ ·6H ₂ O	3.0
K ₂ HPO ₄ ·2H ₂ O	0.03	EDTA	0.5
ASW trace metals	1.0 ml of trace metal stock*		

The pH of the medium was adjusted to 8.0 with concentrated HCl. Where media contained agar double concentrations of ASW and agar were autoclaved separately then mixed together before pouring, to avoid production of toxic substances.

2.4.2 Contamination test medium for *Synechococcus* sp. strains

To test for contamination of *Synechococcus* sp. strains the growth medium (ASW) was enriched with 2% (w/v) glucose, 0.15% (w/v) yeast extract and 1.5% (w/v) Difco bacto-agar. Double concentrations of enriched ASW and agar were autoclaved separately then mixed together before pouring into plates.

2.4.3 Medium for growth of marine heterotrophic bacteria

Marine heterotrophic bacteria were cultured in enriched seawater (ESWIII), based upon seawater agar (Moebus, 1980). ESWIII was made using ASW instead of seawater. To prepare 1 l of ESWIII 250 ml of water was added to 750 ml of ASW, to which were added 5 g of peptone, 1 g of yeast extract and 0.05% (w/v) casamino acids. Plates were made by separately autoclaving double concentrations of ESWIII and 1.5% agar and mixing afterwards, before pouring. Top agar for plaque assays was made similarly, but with a final agar concentration of 0.6% (w/v).

2.4.4 Purification of agar for plates for *Synechococcus* sp. strains

This method has been adapted from Waterbury *et al.* (1986). For *Synechococcus* sp. strains to grow on plates the agar must first be purified. Bacto-agar was washed by stirring 125 g with 3 l of water in a large beaker for 30 min. The agar was allowed to settle, the wash water was poured off and the agar filtered onto 3 MM paper through a large Buchner funnel. This procedure was repeated until the filtrate was clear (typically twice more was adequate). The agar was then stirred with 3 l of ethanol followed by a final wash with 3 l of acetone, stirring each time for 30 min before

settling, pouring off the liquid and filtering. Purified agar was dried at approximately 50°C in a plastic box for 2 days then stored in an air-tight container at room temperature.

2.4.5 Media for growth of *E. coli*

Overnight liquid cultures of *E. coli* were normally grown in nutrient broth; however, for the other growth media used (TYE, M9, top agar and ampicillin plates) see Maniatis *et al.* (1982).

2.5 Culture Conditions

Synechococcus sp. strains were routinely grown in 100 ml batch cultures in 250 ml conical flasks under constant illumination, 5-36 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, at 25°C in ASW, and were incubated without agitation in a Gallenkamp cooled incubator. Once a month stock cultures were subcultured by a 1/10 dilution into fresh medium, and 0.1 ml of the old culture was streaked onto a contamination-test plate which was wrapped in foil and incubated at 25°C for at least a week. Larger volumes were grown in 1 l culture vessels to which 0.5 g l⁻¹ of NaHCO₃ was added. These larger volumes were aerated with filtered air, stirred continuously and maintained at 25°C in a water bath. These vessels were illuminated either from above or from one side by warm-white fluorescent strip lights.

E. coli strain TG1 was stored on M9 plates at 4°C and was restreaked regularly. A single colony was used to inoculate 10 ml of nutrient broth and shaken overnight at 37°C.

2.6 Isolation and Morphological Characterisation of Cyanophages

Surface seawater samples were collected in 1 l plastic bottles and stored at 4°C in the dark. Two 20 ml aliquots were filtered through 0.22 µm into sterile Universals to remove cellular material, and stored at 4°C in the dark.

2.6.1 Clonal isolation and purification of cyanophages

2.6.1.1 Plaque assay

Clonal isolates of cyanophages from seawater samples were obtained by plaque assay followed by plaque purification. A culture vessel containing 1 l of ASW was inoculated with 100 ml of exponentially growing *Synechococcus* sp. strain. This was grown to early-mid-exponential phase ($A_{750} = 0.4$ for strain WH7803), then centrifuged in 250 ml centrifuge tubes at 6000 rpm in a JA-10 rotor for 15 min at 25°C. The supernatant was discarded and the cell pellet resuspended in not more than 25 ml of fresh ASW to give a 40x concentration of cells (*ca.* 8×10^9 cells ml⁻¹). Decimal dilutions of seawater filtrate were added to 0.5 ml aliquots of cell concentrate, and incubated at 25°C for 1 h with occasional, gentle agitation to encourage cyanophage adsorption. Each cyanophage/cell suspension was then added to 2.5 ml of 0.4% (w/v) molten ASW agar (42°C), mixed gently then poured evenly on to a solid 1% (w/v) ASW agar plate (85 mm diameter). Agar used was that which had been especially purified (2.4.4). The plates were then left to set at room temperature for 30 min, inverted then incubated at 25°C under constant illumination, with daily inspection until plaques had formed (generally 3-6 days). Control plates received no cyanophage addition.

Plaque morphotypes were noted and representative plaques were picked using a Pasteur pipette and resuspended in 0.5 ml of ASW. These were then vortexed before storage at 4°C in the dark for at least 12 h to allow cyanophage resuspension. Plaque purification was then achieved by performing a plaque assay on decimal dilutions of the resultant cyanophage resuspension, followed by a final repetition of this procedure to ensure that purified clonal isolates had been obtained.

2.6.1.2 Well assay

Well assays were used as a less labour-intensive method to determine the host-specificity of a cyanophage. Exponentially growing *Synechococcus* sp. cells were concentrated to 5x in a similar manner as for plaque assay. Decimal dilutions of cyanophage were added to 0.2 ml aliquots of the concentrated cells in a 24-well microtitre assay plate. The cyanophage/cell suspensions were incubated at 25°C for 1 h with occasional, gentle agitation, before each sample was diluted with 1.5 ml of ASW. The microtitre plates were incubated at 25°C under constant illumination until host cell lysis was observed (generally within a week). Control wells received no cyanophage addition.

2.6.2 Electron microscopy

Cyanophage clonal isolates were classified morphologically by transmission electron microscopy (TEM).

2.6.2.1 Preparation of grids

A microscope slide was wiped clean with a soft, dry tissue, immersed in a solution of 1% (w/v) formvar in chloroform (stored in a glass stoppered bottle) then left to dry in

the chloroform vapour. A razor blade was used to score the edge of the slide before the slide was slowly lowered obliquely into a beaker of water to float the formvar on the surface. Several copper grids (400 mesh) were placed, all the same face down, onto the formvar film which was then scooped up by placing a sheet of Parafilm on top. The grids were then left to dry under cover from dust and stored at 4°C in a Petri dish.

2.6.2.2 Preparation of specimens

Reversible forceps were used to perforate the formvar around the edge of a coated grid and remove and hold that grid in place. A 20 µl droplet of cyanophage lysate was placed onto the coated surface of the grid and left for 30-60 min until the droplet had half-dried. The remainder was wicked away using filter paper. The grid was immediately washed by placing it sample-side down onto a droplet of deionised water for 30 s, then removing the water with filter paper. The sample was then stained by placing onto a droplet of 1% (w/v) phosphotungstic acid, pH 7.0 (the stock pH adjusted with 10 M KOH) for 1 s, and the excess removed immediately using filter paper. Grids were stored at 4°C until they were viewed using a Joel JEM-100s transmission electron microscope at 80 kV.

2.7 Attempted Isolation of Viruses Infecting Marine Heterotrophic Bacteria

2.7.1 Isolation and purification of marine heterotrophic bacteria

Marine heterotrophic bacteria were isolated in an attempt to isolate bacteriophages which infect them. Decimal dilutions of untreated seawater were spread onto ESWIII

plates and incubated at 25°C for up to two weeks, monitored daily, until a variety of bacteria had grown up. Morphologically distinct colonies were streaked onto individual plates and grown to single colonies, and this purification was repeated twice to obtain axenic stocks.

2.7.2 Attempted bacteriophage isolation

Heterotrophic bacteria which had been isolated were grown in liquid culture in 10 ml of ESWIII in 50 ml flasks, stirring, at 25°C overnight, or until stationary phase had been reached. 10 ml of ESWIII was then inoculated with 0.1 ml of culture and incubated, stirring, at 25°C. Growth curves for the bacteria were obtained by regularly taking readings at A_{550} , to determine the exponential phase.

Plaque assays were then performed on exponentially growing cultures, using seawater filtered through 0.2 μm filters and unfiltered seawater. Decimal dilutions of seawater were added to 200 μl of cells and incubated at 25°C for 30 min with occasional agitation, to allow adsorption. This mixture was then added to 3 ml of 0.6% (w/v) agar ESWIII (at 42°C), mixed gently and poured onto ESWIII plates. Once solidified, these were then inverted and incubated at 25°C overnight, or until a lawn had grown up, and inspected for plaques.

2.8 Molecular Biology Techniques

2.8.1 Cyanophage DNA extraction

2.8.1.1 Extraction from infected cultures

A clonal cyanophage suspension was added to a 50 ml culture of exponentially growing host cells to a multiplicity of infection (MOI) of 4×10^{-3} and incubated at 25°C under constant illumination. Once the culture had lysed (generally within one week) 100 µl of chloroform was added to the lysate, to kill any remaining cells, and DNase and RNase were added to final concentrations of 1 µg ml⁻¹ to degrade any cellular DNA or RNA released during lysis. The lysate was then incubated at room temperature for 1 h before the cell debris was removed by centrifugation at 4500 rpm in a bench-top Wifug Labor-50M centrifuge at 4°C for 10 min. 2% (w/v final concentration) NaCl and 10% (w/v final concentration) polyethylene glycol (PEG) grade 6000 were added to the supernatant, dissolved gently at room temperature and left on ice for 2 h. The precipitated cyanophages were harvested by centrifugation at 4°C for 10 min at 9000 rpm in a JA-20 rotor and resuspended in a total of 1 ml ASW. An equal volume of Tris-HCl (pH 8.0) saturated phenol was added to the cyanophage suspension, vortexed, left for 5 min, vortexed again and centrifuged at 12500 rpm in a bench-top MSE microcentaur for 2 min. The aqueous layer was carefully removed and extracted with an equal volume of Tris-HCl (pH 8.0)-saturated phenol-chloroform (1:1); again this was vortexed and centrifuged before the aqueous layer was removed and finally extracted with an equal volume of chloroform-isoamyl alcohol (24:1). DNA from the resultant aqueous layer was precipitated by adding 0.4 volume of 7.5 M ammonium acetate and 2 volumes of isopropanol. The tube was inverted gently and left at room temperature for 10 min, then DNA was harvested by centrifugation in 1.5 ml Eppendorf tubes for 20 min. The supernatant was discarded and the pellet dried in a desiccator before being resuspended in 40 µl sterile distilled water.

2.8.1.2 Extraction from infected plates

Extraction of cyanophage DNA from infected plates was a faster, more convenient method than DNA extraction from infected cultures, but DNA isolated using this method was not as clean. The plates chosen for DNA extraction were those with confluent plaques caused by the highest titres in a cyanophage plate assay.

Top agar was removed from three ASW agar plates containing confluent lysates of *Synechococcus* spp. cells, mixed with 6 ml ASW containing 1 $\mu\text{g ml}^{-1}$ DNase and 1 $\mu\text{g ml}^{-1}$ RNase and vortexed, prior to incubation at room temperature for 1 h. The top agar was removed by centrifugation at 4500 rpm in a bench-top Wifug Labor-50M centrifuge for 10 min, and the cyanophages were resuspended in the supernatant. DNA was then extracted by the phenol-chloroform method as described for infected cultures.

2.8.2 Extraction of DNA from seawater

In order to perform PCR on seawater samples to detect cyanophages, their DNA was initially extracted. To 0.5 ml of seawater an equal volume of Tris-HCl (pH 8.0)-saturated phenol-chloroform (1:1) was added. This was vortexed, left for 5 min, vortexed again and centrifuged for 2 min at 12500 rpm in a bench-top MSE microcentaur. 450 μl of supernatant was removed into a clean Eppendorf tube, to which was added 0.4 volume of 10.5 M ammonium acetate, 2 volumes of ethanol and 2 μl of 10 mg ml^{-1} glycogen to help form a DNA pellet. This was mixed and incubated at -20°C overnight before centrifuging for 20 min, washing the pellet with 0.5 ml of 70% (v/v) ethanol, centrifuging for 5 min and desiccating for 10 min. The DNA pellet was resuspended in 20 μl of sterile, distilled water.

2.8.3 Restriction endonuclease digestion

DNA was routinely cut by restriction endonuclease digestion. The digest reaction mixture comprised spermidine (10x stock of 40 mM), RNase (DNase-free, 20x stock of 10 $\mu\text{g ml}^{-1}$), restriction enzymes and their respective buffers. Digestions were incubated according to the manufacturers' instructions (usually 37°C for 2 h).

2.8.4 Agarose gel electrophoresis

Agarose gels for separating DNA fragments were made up in TBE. 0.7% (w/v) gels were used for routine DNA electrophoresis and ethidium bromide was added to a final concentration of 0.5 $\mu\text{g ml}^{-1}$. Gels were run at 70 mA for 2 h in 1x TBE running buffer. Loading buffer (0.1 volume) was added to the digest reaction mixture before loading. DNA fragments were observed with long-wave UV light. Photographs were taken using a Polaroid camera and short-wave UV light.

Excision of DNA fragments from agarose gels was performed by either of two methods, depending on the size of the DNA fragment, following excision of the fragment using a clean scalpel blade. For fragments >400 bp the GeneClean II kit was used, following the instructions supplied by the manufacturer. For fragments <400 bp the QIAquick Gel Extraction kit was used, following the instructions supplied by the manufacturer.

2.8.5 Construction of a recombinant plasmid

2.8.5.1 Dephosphorylation of pUC19 vector DNA

pUC19 (Yanisch-Perron *et al.*, 1985) vector DNA was restricted with the same restriction endonuclease used to restrict clonal cyanophage DNA. Once digestion was

complete 0.1 volume of 10x dephosphorylation buffer (0.5 M Tris-HCl pH 8.5, 1 mM EDTA), supplied by the manufacturer, Boehringer Mannheim, and 2 units of alkaline phosphatase (from calf intestine, CIP) were added and the sample was incubated at 37°C for 30 min. A further 2 units of CIP were added and incubated for 30 min. The preparation was run into a 0.7% (w/v) agarose gel and the dephosphorylated pUC19 DNA was recovered from the gel using a GeneClean II kit.

2.8.5.2 Ligation of foreign DNA into pUC19 vector

Different quantities of restricted and cleaned cyanophage DNA were used in each reaction to ensure an efficient ligation yield. Ligation reactions were typically prepared as follows but amounts of vector and insert were varied depending on concentrations observed on an agarose gel, in order to have an excess of insert compared with vector:

vector DNA	2 µl
insert DNA	6 µl
10 mM ATP	2 µl
5x ligase buffer	4 µl
T4 DNA ligase	1 µl
water	5 µl

Usually at least two ligations were set up with different insert/vector ratios, and a control reaction with no insert. If "sticky-end" ligations were performed then the vector, insert and water were heated to 65°C for 10 min and cooled on ice before adding the remaining constituents. Reaction mixes were incubated at 15°C overnight.

2.8.5.3 Preparation of competent cells for transformation

An overnight *E. coli* TG1 culture was diluted 1:50 in 50 ml of TYE and incubated at 37°C in an orbital incubator until an A_{600} of 0.92-0.96 was reached, when it was transferred to ice for 10 min. The cells were concentrated by centrifugation in Universals at 4500 rpm in a bench-top Wifug Labor-50M centrifuge for 5 min at 4°C, then resuspended in 20 ml of ice-cold 0.1 M CaCl_2 and incubated on ice for at least 20 min prior to centrifugation under the same conditions. The resultant pellet was resuspended in enough ice-cold 0.1 M CaCl_2 for 200 μl per ligation mix and incubated on ice until transformation (not >24 h).

2.8.5.4 Transformation

Competent cells were divided into aliquots of 200 μl and 10 μl of a vector/insert DNA ligation mix was added to each aliquot and left on ice for 2 h with occasional agitation. The competent cells/ligation mixes were "heat-shocked" at 42°C for 3 min before adding 1 ml of TYE broth and incubating at 37°C for 1 h with occasional agitation. Cells were then concentrated in a bench-top microfuge, resuspended in 50 μl of the supernatant and spread on to a TYE plate containing 50 $\mu\text{g ml}^{-1}$ ampicillin. For detection of β -galactosidase activity (indicating transformants without inserts) TYE ampicillin plates containing 50 μl of 2% (w/v) XGal in DMF and 50 μl of 2% (w/v) IPTG were used for plating cells. After overnight incubation, at 37°C, "white" colonies were picked and streaked onto a fresh TYE plate containing 50 $\mu\text{g ml}^{-1}$ ampicillin and incubated at 37°C for at least 6 h. Cells were picked into TYE broth containing 50 $\mu\text{g ml}^{-1}$ ampicillin and incubated overnight, with constant shaking, prior to isolation of plasmid DNA. If, during the generation of a library, a large number of colonies was being screened for the presence of a particular DNA insert, colonies were then grouped together after the initial incubation to reduce the effort in DNA preparation and screening.

2.8.6 Purification of plasmid DNA from *E. coli*

Mini preparation of plasmid DNA from 1.5 ml from an 10 ml overnight culture was performed. The cells were concentrated in Eppendorf tubes by centrifugation at 12500 rpm in a bench-top MSE microcentaur for 1 min, resuspended in 100 μ l of ice cold GTE containing 10 mg ml⁻¹ lysozyme and stored on ice for 5 min. 200 μ l of 0.2 M NaOH, 1% (w/v) SDS (freshly prepared) was added and the tube was inverted until the contents cleared, to ensure complete cell lysis. The sample was then placed on ice for 5 min before adding 150 μ l of ice cold potassium acetate pH 4.8, vortexing gently and then storing on ice for 5 min. The precipitate was removed by centrifugation for 5 min and the supernatant was transferred to a fresh Eppendorf tube where it was extracted with 1 volume of phenol/chloroform by vortexing, leaving for 2 min and vortexing again before centrifuging for 5 min. The aqueous phase was removed into a clean tube and precipitated by adding 2 volumes of ethanol, incubating at -20°C for 2 h, then centrifuging for 10 min. The plasmid DNA pellet was washed in 70% (v/v) ethanol, dried in a desiccator for 5 min and resuspended in 30 μ l of TE.

2.8.7 Spectrophotometric determination of DNA concentration

DNA was diluted 1 in 100 with distilled water in a quartz cuvette of 1 cm pathlength. The absorbance was read at 260 nm and 280 nm, measuring against a distilled water blank in an LKB Ultrospec spectrophotometer. The DNA concentration was determined on the assumption that an A_{260} of 1.0 is equivalent to 50 μ g ml⁻¹ of double-stranded DNA. For single-stranded DNA, A_{260} of 1.0 is equivalent to 20 μ g ml⁻¹. The ratio of A_{260}/A_{280} gave an indication of the purity of the DNA sample, an A_{260}/A_{280} ratio of 2 indicated pure DNA with no contaminating proteins. Typically the value was between 1.5 and 1.9.

2.8.8a Southern blotting (method 1: radioactive labelling)

Table 2.5 Reagents used for radioactive Southern hybridisation.

Reagent	Composition
Denhardts (50x)	1 g Ficoll, 1 g PVP, 1 g BSA in 100 ml of water. The solution was filter sterilised and stored at 4°C.
20x SSC	175.3 g NaCl, 88.2 g sodium citrate in 800 ml water, adjusted to pH 7 then made up to 1 l with water.
20x SSPE	174 g NaCl, 31.2 g NaH ₂ PO ₄ .H ₂ O, 7.4 g EDTA.Na ₂ in 800 ml water, adjusted to pH 7.4 with 10 M NaOH (approximately 21 ml), made up to 1 l with water.

2.8.8a.1 Blotting procedure

Restriction digests of the required DNA were run on a 0.7% (w/v) agarose gel. After electrophoresis the gel was photographed with rulers for scale. The gel was washed in the following solutions (500 ml): 0.25 M HCl for 10 min; 2 washes of 0.5 M NaOH, 1.5 M NaCl for 15 min each wash; and finally 2 x 15 min washes of 1 M Tris-HCl pH 7.4, 70 ml concentrated HCl, 3 M NaCl.

The blot was constructed as described in Maniatis *et al.*, (1982), with 10x SSC buffer, and left overnight, after which the dehydrated gel was discarded and the nitro-cellulose filter was soaked in 6x SSC at room temperature for 5 min before being dried on filter paper. The filter was then baked at 80°C between two sheets of 3 MM paper, under vacuum, for 1 h.

2.8.8a.2 Prehybridisation of filters

The 3 MM paper containing the baked nitro-cellulose filter was placed in a shallow tray of 2x SSC before removing the filter which was overlaid on a nylon mesh and placed in a glass bottle with 20 ml of preheated prehybridisation fluid (5x SSPE, 5x Denhardts and 0.1% (w/v) SDS). This was then placed in a Hybaid hybridisation

oven, according to the manufacturers instructions, at the hybridisation temperature for at least 4 h.

2.8.8a.3 Preparation of probes

Purified DNA probe was placed in a boiling water bath for 3 min to denature the DNA, then kept at 37°C for 10 min. To label the probe a reaction was prepared, containing 5 µl of OLB buffer (Table 2.6), 1 µl of BSA (10 mg ml⁻¹), 16 µl of DNA fragment (25 ng), 2.5 µl of ³²P dCTP (10 µCi µl⁻¹) and 0.5 µl of Klenow fragment of DNA polymerase I. This was incubated behind a radioactive screen at room temperature for at least 5 h.

Table 2.6 Reagents used in radioactive labelling reaction.

Reagent	Composition
solution O	1.25 M Tris-HCl, 0.125 M MgCl ₂ , pH 8 (stored at 4°C).
dNTPs	dATP, dTTP, dGTP, each 0.1 M in TE buffer (stored at -20°C).
solution A	1 ml solution O, 18 µl 2-mercaptoethanol, 5 µl each of dATP, dTTP and dGTP.
solution B	2M HEPES pH 6.6 (pH adjusted with 4 M NaOH, stored at 4°C)
solution C	Hexadeoxynucleotides (Pharmacia, PL 2166). 50 absorbance units were suspended in 550 µl TE buffer to give a concentration of 90 absorbance units ml ⁻¹ (stored at -20°C).
OLB Buffer	Solutions A, B and C were mixed in the ratio 10:25:15 (stored at -20°C in 50 µl aliquots).

When the labelling reaction was complete, 100 µl of TE and 100 µl of herring sperm DNA (10 mg ml⁻¹) were mixed and added to the labelled probe before it was heated in a boiling water bath for 10 min then placed on ice for 5 min.

2.8.8a.4 Hybridisation and washing

After prehybridisation the fluid was poured off and 9.5 ml of fresh hybridisation fluid (same as prehybridisation fluid) was added to the bottle. 0.5 ml of hybridisation fluid

was added to the labelled probe and mixed, and this was then added to the bottle before incubating at the required temperature in the Hybaid oven for 12-24 hours.

After hybridisation the filter was given increasingly stringent washes depending on the homology of the probe, starting with 2x SSPE and 0.1% (w/v) SDS at room temperature. The most stringent wash, for probes with the highest homology to target DNA, was 0.1x SSPE, 0.1% (w/v) SDS at 70°C for 30 min. Filters were checked with a Geiger counter for the level of background counts. On the completion of the wash, the filter was placed between two pieces of plastic inside a Harmer X-ray cassette. An intensifying screen was placed on top of the X-ray film and the cassette was left at -70°C until ready to be developed.

2.8.8a.5 Stripping blots

Hybridised probe was removed from a blot by boiling 1.5 l of 0.1% (w/v) SDS, turning off the heat and placing the nitro-cellulose filter in the SDS until it had cooled to room temperature. The filter was then stored moist in a sealed plastic bag.

2.8.8b Southern blotting (method 2: non-radioactive labelling)

A safer alternative to the use of ^{32}P for Southern hybridisation is the use of DIG-oxygenin. The blotting procedure is the same as for method 1; however, nylon filters were preferentially used, which were fixed after blotting, by covering in cling-film and placing on a short-wave UV box for 3 min. The procedures for prehybridisation, preparation of the probe, hybridisation and detection of the probe (using the colorimetric alkaline phosphatase method) were all followed according to the instructions of the manufacturer.

Used probe, already in 20 ml of hybridisation fluid, can be reused by denaturing at 68°C for 15 min before adding directly to the prehybridised filter in the glass hybridisation bottle. The probe can be stored at -20°C and reused several times.

After hybridisation the filter was given increasingly stringent washes depending on the homology of the probe, starting with two washes for 5 min with 2x SSC and 0.1% (w/v) SDS at room temperature. The most stringent wash, for probes with the highest homology to target DNA, was two washes for 15 min with 0.1x SSC and 0.1% (w/v) SDS at 70°C for 30 min.

A more rapid method was occasionally used, in which the filter was prehybridised for 30 min then hybridised to the probe for 6 h. Stringency washes, with the above constituents, were then once at room temperature for 10 min followed by once at the hybridisation temperature for 15 min.

2.8.9 DNA sequencing (manual)

Manual sequencing was performed using the dideoxy chain terminator/M13 vector method (Sanger *et al.*, 1977 and Messing *et al.*, 1977).

Procedures 2.8.9.1 to 2.8.9.4 were carried out for random subcloning into M13. To clone specific fragments with specific "sticky" ends the restricted DNA was run on an agarose gel, excised, purified using the GeneClean II kit, and ligated directly into replicative-form M13 DNA which had been restricted with the appropriate endonucleases.

Table 2.7 Reagents used for sequencing.

Reagent	Composition
25% (w/v) AMPS	25% (w/v) ammonium persulphate in water, stored at 4°C.
Formamide dye mix	100 ml formamide (deionised with mixed bed resin), 0.1 g xylene cyanol, 0.1 g bromophenol blue, 2 ml 0.5 M EDTA pH 8.
10x Ligase buffer	500 mM Tris-HCl pH 7.5, 100 mM MgCl ₂ , 100 mM dithiothreitol (DTT).
Sequenase buffer	100 mM Tris-HCl, pH 8.5, 50 mM MgCl ₂ .
XGal	2% (w/v) 5-bromo-4-chloro-3-indolyl-galactoside in DMF.
IPTG	2.5% (w/v) isopropyl-d-thiogalactopyranoside in water.
40% (w/v) acrylamide	380 g acrylamide, 20 g NN-methylenebisacrylamide. Volume brought to 1 l with deionised water; stirring gently with 20 g mixed bed resin. Filtered and stored at 4°C.
0.5x TBE 6% (w/v) gel mix	75 ml 40% (w/v) acrylamide, 25 ml 10x TBE, 230 g urea. Volume brought to 500 ml with deionised water. Filtered and stored at 4°C.
5x TBE 6% (w/v) gel mix	30 ml 40% (w/v) acrylamide, 100 ml 10x TBE, 92 g urea, 10 mg bromophenol blue. Volume brought to 200 ml with deionised water. Filtered and stored at 4°C.
dNTP chase	0.5 mM each of dTTP, dCTP, dGTP and dATP from 50 mM stocks in TE.
5x labelling mix	7.5 µM each of dGTP, dCTP and dTTP from 10 mM stocks in TE. Stored in 10 µl aliquots.
ddG termination mix	80 µM each of dGTP, dATP, dCTP and dTTP; 8 µM ddGTP; 50 mM NaCl
ddA termination mix	80 µM each of dGTP, dATP, dCTP and dTTP; 8 µM ddATP; 50 mM NaCl
ddC termination mix	80 µM each of dGTP, dATP, dCTP and dTTP; 8 µM ddCTP; 50 mM NaCl
ddT termination mix	80 µM each of dGTP, dATP, dCTP and dTTP; 8 µM ddTTP; 50 mM NaCl

2.8.9.1 Fragment self-ligation

The fragment of DNA to be sequenced was recovered from an agarose gel, using a GeneClean II kit, prior to resuspension in 20 µl of water to which 3 µl of 10x ligase buffer, 3 µl of 10 mM ATP, 2 µl of water and 2 µl of T4 DNA ligase were added. The ligation mixture was then incubated at 15°C for 2-3 h.

2.8.9.2 Sonication

Once fragment self-ligation was complete the Eppendorf containing the sample was placed in a W-380 ultrasonic processor (Heat Systems-Ultrasonics Inc., Farmingdale, USA) and the sonicator tank was filled with water to a level 10 mm above the level of the digest. Full power was engaged for 60 s, then the whole process was repeated with fresh water in the tank after the sample had been briefly centrifuged in a bench-top MSE microcentaur.

2.8.9.3 Fragment end repair

The self ligated and sonicated fragment in 30 μ l was mixed with 2 μ l of dNTP chase, 2.5 μ l of Klenow fragment of DNA polymerase I and 2.5 μ l of T4 DNA polymerase, then incubated at room temperature for 3-4 h.

2.8.9.4 Fragment size fractionation

End-repaired fragments were loaded on to a 1% (w/v) agarose gel and the gel was run at 60 mA until the bromophenol blue marker dye had migrated 2 cm. The size range required was recovered from the gel by excising and extracting the DNA using the GeneClean II kit.

2.8.9.5 Ligation into M13

The M13 vector was prepared by cutting M13 DNA with the appropriate endonuclease then dephosphorylating and purifying the linear DNA as described previously (2.8.5.1; 2.8.4). Different quantities of fragments were used in each ligation reaction to ensure an efficient yield. Ligation reactions were typically performed in like manner to ligation of foreign DNA to pUC19 vector (2.8.5.2).

2.8.9.6 Preparation of competent cells for transfection

An overnight *E. coli* TG1 culture was diluted 1:100 in 30 ml TYE and incubated at 37°C until an A₆₀₀ of between 0.4 and 0.6 was reached, before it was left on ice for 30 min. Cells were concentrated by centrifugation in Universals at 4500 rpm in a Wifug Labor-50M centrifuge for 5 min at 4°C, before the pellet was resuspended in 20 ml of ice-cold 0.1 M MgCl₂ then incubated on ice for 20 min before being

concentrated again by centrifugation at 4°C. Cells were then resuspended in 20 ml of ice-cold CaCl₂ and incubated at 4°C for 20 min prior to centrifugation under the same conditions. The resultant pellet was resuspended in a total of 1 ml of ice-cold 0.1 M CaCl₂ and left on ice for at least 2 h.

2.8.9.7 Transfection

The competent cells were divided into 200 µl aliquots and 10 µl of a fragment/vector ligation was added to each one. The samples were then left on ice for 45 min with occasional agitation. The competent cells/ligation mixes were "heat shocked" at 42°C in a water-bath for 3 min before promptly being added to sterile culture tubes, each containing 3 ml of molten top agar (42°C), 25 µl of 2% (w/v) XGal and 25 µl of IPTG. Without any delay each tube of agar was mixed briefly and poured onto a TYE plate. The agar was left to set for 15 min prior to an overnight incubation at 37°C.

2.8.9.8 Template preparation

A toothpick was used to pick a white plaque from the transfection plate and placed in a sterile culture tube containing 1.5 ml of an overnight TG1 culture diluted by 1:100 in TYE. This was grown with vigorous shaking (>400 rpm) at 37°C for 5 h. The culture was then transferred to a 1.5 ml Eppendorf tube and concentrated by centrifugation in a bench-top MSE microcentaur. Care was taken not to carry over any cells as the supernatant was transferred to a fresh Eppendorf tube. 200 µl of 20% (w/v) PEG, 14.6% (w/v) NaCl was added to the supernatant which was then vortexed and left to incubate at room temperature for at least 10 min before it was centrifuged. The supernatant was removed and the pellet desiccated for 10 min before resuspending it in 100 µl of TE. 1 volume of Tris-HCl (pH 8.0) saturated phenol was added, vortexed, left for 5 min, vortexed again and centrifuged for 2 min.

Approximately 90 μ l of the aqueous layer was removed (taking extreme care not to remove any of the interface), to which 9 μ l of 3 M sodium acetate and 225 μ l of ethanol were added before precipitating the DNA at -20°C for 2 h. The pellet obtained after centrifuging for 20 min, washing with 70% (v/v) ethanol and desiccating was dissolved in 30 μ l of TE.

2.8.9.9 Preparation of a buffer gradient gel for sequencing

The sequencing gel was made as follows: 45 ml of 0.5x TBE 6% (w/v) gel mix was added to one beaker and 7 ml of 5x TBE 6% (w/v) gel mix to another beaker. Polymerisation was initiated first by adding 80 μ l (to the 0.5x) and 14 μ l (to the 5x) of 25% (w/v) AMPS, then 80 μ l (0.5x) and 14 μ l (5x) of TEMED. 30 ml of the 0.5x gel mix was promptly taken up in a syringe (and put to one side) before 8 ml was taken up in a 25 ml pipette followed by all of the 5x gel mix. A rough gradient was formed by allowing two to three air bubbles to pass up through the pipette. The gradient was poured down the inner edge of the previously taped-together gel plates (48 cm x 20 cm), holding them at an angle of approximately 45°. The 0.5x gel mix remaining in the syringe was added next, maintaining an even flow until the glass plates were filled, then the slot former was put quickly in place before the plates were clamped together and left, with the open end slightly raised, until the gel had polymerised.

2.8.9.10 Sequenase sequencing

Sequencing reactions were carried out using a Sequenase version 2.0 kit (USB) according to the manufacturers instructions. Just before loading the sequencing reactions, the buffer gradient gel was clamped onto the electrophoresis apparatus and the top and bottom tanks were filled with 1x TBE which was used to flush out the slots and acted as the running buffer. Each sample was then loaded onto the gel and run at 37 W for 3 h. After the power was disconnected the silanised glass plate was

gently lifted off, exposing the gel, which was then placed into a solution of 10% (v/v) acetic acid for 15 min to fix the gel. Once the gel was fixed it was drained before being transferred to a sheet of 3 MM paper, covered with a sheet of cling film then placed in a gel drier where it was dried under vacuum at 80°C for 45 min or until dry. The dried gel was transferred to a film cassette after removing the cling film, an X-ray film was placed directly on top of it and left at room temperature overnight before it was developed.

2.8.9.11 Analysis of sequence data

Sequence data from autoradiograms were read into "MicroGenie", a sequence analysis program (after Queen and Korn, 1984), then transferred to GCG (Genetics Computer Group), a sequence analysis software package (Devereux *et al.*, 1984) available on the departmental computer network. Sequence data could then be analysed and compared with characterised sequences in databases within GCG.

2.8.10 Polymerase chain reaction

2.8.10.1 PCR conditions and gel electrophoresis

Optimisation of PCR resulted in the following conditions being used for the set of primers designed (CPS1 and CPS2):

50 mM MgCl ₂	10 µl
10x PCR buffer	5 µl
2.5 mM dNTPs	4 µl
1 µM CPS1	2 µl
1 µM CPS2	2 µl
target DNA	1 µl
sterile water	26 µl

The above constituents were mixed in 0.65 ml siliconised tubes and overlaid with 50 μ l of paraffin. For multiple samples, especially when used for quantification, a master mix containing all the components except the target DNA, was made, to minimise tube-to-tube differences (see Romanowski *et al.*, 1993). The PCR was performed in a Perkin-Elmer thermal cycler with a "hot start" of 94°C for 5 min followed by holding the temperature at 80°C, during which time 0.25 μ l of 5 Units μ l⁻¹ *Taq* polymerase was added to the reaction mixture. The thermal cycler then went through 35 cycles of: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min.

PCR products were visualised by gel electrophoresis. Typically 15 μ l aliquots of product were loaded into 1x TBE, 1.2% (w/v) agarose gels containing 0.5 μ g ml⁻¹ ethidium bromide, using 0.1 volume of loading buffer. Gels were run routinely at 60 mA for 1 h in 1x TBE running buffer, in the same tanks, and with the same visualisation method, as for separation of restricted DNA fragments (2.8.4).

2.8.10.2 DNase treatment of cyanophage lysates

In order to remove free cyanophage DNA from a lysate prior to virus quantification by PCR it was necessary to treat the lysate with DNase. To 10 μ l of decimal dilutions of lysate DNase was added to 1 μ g ml⁻¹ and incubated at room temperature for 30 min (for DNase treatment of DNA in water it was necessary to add MgCl₂ to 4.2 mM). The PCR reaction was prepared as normal, but the primers were added during the "hot start" at 80°C, just before addition of *Taq* polymerase.

2.8.10.3 PCR product quantification by laser densitometry

The negative image from a Polaroid photograph of PCR products run on an agarose gel was scanned by a scanning laser densitometer. The intensity of bands

representing individual PCR products was consequently quantified using Image Quant (version 3.3) software.

2.8.11 Sequencing PCR products

Fresh PCR products were ligated into the TA cloning vector pCR2.1 using the Invitrogen Original TA Cloning Kit. The procedure for ligation and transformation was followed according to the instructions of the manufacturer, using the competent cells provided in the kit.

2.8.11.1 Screening transformants for presence of PCR product

Transformant colonies were screened for the possession of the PCR product by touching the top of the colony very gently with a tooth pick and transferring these cells to 50 µl of sterile water. This cell suspension was heated to 94°C for 5 min to break open the cells, and PCR was performed on 1 µl of this.

2.8.11.2 Rapid mini and CsCl preparation of plasmid DNA

Transformant colonies containing PCR products were inoculated into 5 ml of TYE with 50 µg ml⁻¹ of ampicillin and cultured overnight at 37°C. The cells were concentrated from 4 ml by centrifugation at 12500 rpm in a bench-top MSE microcentaur for 1 min and resuspended in 100 µl of GTE. From then on the procedure from Saunders and Burke (1990) was followed.

2.8.11.3 Preparation of DNA for automated sequencing

To 5 μl of CsCl-pure DNA, 0.5 μl of 2 M NaOH and 0.5 μl of 2 mM EDTA were added to denature the DNA. This was incubated at 37°C for 30 min before neutralising with 0.6 μl of 3 M NaOAc pH 4.5-5.5. The DNA was precipitated by adding 20 μl of ethanol and incubating at -70°C for 15 min. The DNA was centrifuged for 20 min at 12500 rpm in a bench-top MSE microcentaur and the pellet washed in 70% (v/v) ethanol before air-drying for 5 min and resuspending in 10.2 μl of sterile water. To this, 1.8 μl of 10 $\mu\text{g ml}^{-1}$ sequencing primer was added, giving a final volume of 12 μl sent to be sequenced.

2.8.12 Separation of same-sized PCR products

PCR products of the same size were separated using the chemical HA-Yellow (HA-Y) from Hans Analytik. After optimisation of this technique products were run on a 0.5x TBE, 3% (w/v) agarose gel containing 1 unit ml^{-1} of HA-Y. The HA-Y was added after melting the agarose, when it had cooled to 70°C. No ethidium bromide was added. The products were run using 0.5x TBE buffer and at 14 V cm^{-1} (148 V for a 10.5 cm-long gel). Both gel and buffer were precooled to 4°C and during the electrophoresis the buffer was continually circulated through an ice bucket to keep it cool, and to prevent any pH gradients from forming. DNA fragments could be visualised faintly with short-wave UV to check migration, then the gel was placed in 200 ml of 0.5x TBE containing 0.5 $\mu\text{g ml}^{-1}$ of ethidium bromide for 30 min to stain the DNA, before visualising and photographing using short-wave UV.

2.9 Counting *Synechococcus* spp.

To count *Synechococcus* spp. in marine samples 20-200 ml of seawater was filtered through a 0.2 μm pore-size membrane with a 0.45 μm pore-size backing membrane,

using a suction pump. The former membrane was then placed on a drop of glycerol on a microscope slide, and another drop placed on top of the membrane before pressing a cover slip down on top. Cells were then visualised and counted using a UV epifluorescence microscope with a green filter; *Synechococcus* cells fluoresced orange. Counts were determined from the average of 10-30 fields of view, depending on the concentration of cells present.

2.10 Counting Free Viruses by Light Microscopy

For rapid quantification of the titre of stock cyanophage lysates virus particles were counted using the nucleic acid dye TOTO-1 from Molecular Probes Inc..

Cyanophage stocks were decimally diluted and 10 µl samples diluted in 0.5 ml of ASW. To this, 0.5 ml of sterile, distilled water was added. The TOTO-1 dye stock was diluted 1/10 in PBS, and 5 µl of this dilution was added to the diluted cyanophages before incubating overnight at room temperature in the dark. The stained cyanophages were then filtered on to 0.02 µm pore-size membranes with a 0.45 µm pore-size backing membrane, using a suction pump. The former membrane was then mounted on a slide as in section 2.9. Cyanophages were visualised and counted using a UV epifluorescence microscope with a blue filter; cyanophages fluorescing as small, bright, yellow dots against a black background. Counts were determined from the average of 10-30 fields of view, depending on the concentration of cyanophages present.

2.11 Concentration of Viruses from Seawater

Seawater samples collected on the AMT-2 cruise were collected at varying depths from 7-150 m in 10 l Go-Flow bottles, from which 1 l samples were taken in polycarbonate bottles. The Go-Flow bottles were deployed on a sampling rosette with CTD (conductivity, temperature, depth) apparatus.

2.11.1 Tangential flow filtration

1 l seawater samples were prefiltered through 2 μ m pore-size membranes to remove larger phytoplankton and zooplankton. These filtrates were then concentrated to 5 ml using a Verder peristaltic pump and a Mini-Ultrasette from Flowgen, with a 30 kDa cut-off point, so that cyanophages were retained in the concentrate. The 5 ml concentrate was placed in a plastic Universal and 9 ml of filtrate was used to wash out the residual cyanophages from within the tubing and Mini-Ultrasette, into the same Universal. Samples were then quick-frozen and stored at -60°C. Samples were thawed on ice before use.

The Mini-Ultrasette was washed between samples first with 500 ml of distilled water passing across the membrane, not through it. Next, 200 ml of 1 M NaOH was recirculated through the Mini-Ultrasette for 15 min, allowing at least 50 ml to filter through. Next, 200 ml of 1 M HCl was recirculated similarly. Finally, 500 ml of filtrate from a previous sample was passed across the membrane, allowing at least 50 ml to filter through. The Mini-Ultrasette was soaked overnight in distilled water.

Attempts were made to further concentrate the cyanophages using the methods outlined below. These methods were attempted only on ASW samples with known quantities of cyanophages added.

2.11.2 Ultracentrifugation

Samples of 10 ml of ASW containing cyanophages was placed in a 14 ml SW40 ultracentrifuge tube and ASW was added to about 0.5 cm from the top of the tube. Tubes were balanced to within 0.002 g and centrifuged at 30000 rpm at 4°C for 1 h, using an SW40Ti rotor. About 13 ml of supernatant was carefully removed and the cyanophage pellet was resuspended in the remaining 0.5 ml and stored at 4°C.

2.11.3 Polyethylene glycol precipitation

Cyanophage dilutions were added to 0.5 ml of ASW, to which NaCl was added to 2% (w/v) and dissolved. PEG was then added to 10% (w/v) and dissolved, and 2 μ l of 10 mg ml⁻¹ glycogen was added to help form a pellet before leaving for 1 h-overnight at 4°C. Samples were then centrifuged at 12500 rpm in a bench-top MSE microcentaur for 20 min and the pellets were desiccated for 10 min before being resuspended in 10 μ l of sterile distilled water to reduce salt concentration for PCR.

2.11.4 Zinc chloride precipitation

Cyanophage concentration was attempted by precipitating with zinc chloride, based on the method described by Santos (1991). To the cyanophage dilution in 1 ml of ASW, ZnCl₂ was added to 40 mM (10 μ l of filter-sterile 4 M stock) and 2 μ l of 10 mg ml⁻¹ glycogen was also added, before the sample was incubated at 37°C for 5 min. The sample was then centrifuged at 12500 rpm in a bench-top MSE microcentaur for 1 min and the pellet desiccated for 10 min before resuspending in 10 μ l of sterile distilled water.

Chapter 3:

Isolation and Characterisation of Novel Marine Viruses

3.1 Introduction

Marine cyanophages infecting marine phycoerythrin-containing *Synechococcus* spp., which had previously been isolated from a range of different geographical locations, were found to exhibit DNA homology by Southern hybridisation (Wilson *et al.*, 1993). An attempt was made to isolate yet more cyanophages from additional locations to obtain a fuller picture of the global marine cyanophage population, and to provide a useful collection for the subsequent development of molecular techniques to analyse cyanophage populations. These molecular techniques would be based on the DNA region which was found by Wilson *et al.* (1993) to be conserved amongst these cyanophages. This same region was also found to be partially homologous to gene 20 of the coliphage T4 (Wilson, 1994). This gene encodes a structural tail tube protein (gp20), which is also responsible for initiation of head assembly, DNA packaging and binding with the tail connector (accession number P13334; Arisaka *et al.*, 1988; Marusich and Mesyanzhinov, 1989).

Morphological and host range characterisation of the cyanophages was necessary to compare with subsequent molecular analysis. Preliminary molecular characterisation was performed by probing digests of the novel cyanophage DNA with the conserved region from cyanophage strain S-PM2, to determine whether there was any homology. For the development of molecular techniques it was also necessary to obtain non-cyanophage marine viruses to act as suitable controls. Hence, attempts were made to isolate viruses which infect marine heterotrophic bacteria.

For the isolation of cyanophages the host strain used was primarily *Synechococcus* sp. WH7803. It is known that cyanophages exhibit some degree of host strain specificity (Suttle and Chan, 1993; Waterbury and Valois, 1993), such that the use of a single strain to isolate cyanophages would result in a proportion of natural cyanophages not being detected. However, *Synechococcus* sp. WH7803 has been shown to be susceptible to infection by a wide range of cyanophages (Waterbury and Valois, 1993), and so appears to be a suitable candidate for cyanophage isolation.

Synechococcus sp. WH8018 was also used, where cyanophages had already been detected, to isolate any cyanophages which might not infect *Synechococcus* sp. WH7803. These two host strains differ in phycoerythrin content and place of isolation (Waterbury, *et al.*, 1986). *Synechococcus* sp. WH7803 was isolated from the open ocean (Sargasso Sea), whereas *Synechococcus* sp. WH8018 was isolated from the east coast of the USA (Woods Hole). *Synechococcus* sp. WH8103 was used for host range studies, though not for cyanophage isolation, since its susceptibility to infection is more limited (Waterbury and Valois, 1993), and this strain did not grow easily on agar plates for plaque purification of cyanophages.

It has been shown that marine bacteriophages are rapidly degraded in the natural environment, largely from sunlight, particles larger than 0.22 μm , and dissolved organic matter (*e.g.* Suttle and Chen, 1992; Noble and Fuhrman, 1997; see 1.3.3.3). To reduce cyanophage decay in the samples which were collected, the water was filtered through 0.22 μm pore-size membranes and stored in the dark at 4°C as soon as possible.

Cyanophage isolation was achieved by plaque formation on a lawn of the host, grown on solid medium (2.6.1.1). This same method was used to purify cyanophages. The plaque formation method was adopted in preference to lysis of liquid cultures (2.6.1.2) since it is a more accurate method for counting viable cyanophages and clearly separates different cyanophages as a first step of purification.

3.2 Isolation and Plaque Purification of Cyanophages

Seawater samples were obtained from various different geographical locations; many were kindly collected by friends on holiday. The water was collected in *ca.* 100-500 ml polyethylene bottles from coastal surface waters and kept cool and dark before

two 20 ml aliquots were filtered through 0.22 µm pore-size membranes in the laboratory, and stored, together with the untreated samples, at 4°C in the dark.

Seawater was collected from coastal waters off: Barbados; Bermuda; Cape Trafalga, SW Spain; Gambia; west Gibraltá; Helsinki, Finland; Lesbos, Greece; Mauritius; Plymouth; San Pedro, SE Spain; and Trinidad. Seawater was also collected from the open ocean site, Hydrostation S in the Sargasso Sea. Samples collected from Barbados, Bermuda, Hydrostation S and Trinidad were all filtered on site. Seawater samples from coastal Plymouth and San Pedro were filtered two days after collection. All other samples (except from coastal Helsinki) were filtered a few days after collection. Plaque isolation (2.6.1.1) was attempted from each of these samples (except the samples from Helsinki and Trinidad). However, plaques were not obtained from any of the unfiltered seawater samples.

At Hydrostation S (32° 4' N, 64° 23' W) water was collected from 1 m depth, using the research vessel Weatherbird II, from Bermuda Biological Station for Research (BBSR). The following day plaque isolation was attempted (2.6.1.1), using *Synechococcus* sp. WH7803 from BBSR, on dilutions of the filtered water. A titre of *ca.* 10 PFU ml⁻¹ (not statistically reliable) was obtained. Total *Synechococcus* spp. counts were also made (2.9); which were *ca.* 6 x 10² ml⁻¹. Plaque diameter ranged from 1-4 mm (after one week), and two representative plaques were picked and later plaque-purified (2.6.1.1) using *Synechococcus* sp. WH7803 from Warwick.

Four weeks later plaque isolation (2.6.1.1), using *Synechococcus* sp. WH7803 from Warwick, was attempted on seawater from Hydrostation S. This resulted in no plaques. However, seawater from coastal Bermuda, collected about 20 m offshore and 1 m depth, showed a titre of *ca.* 100 PFU ml⁻¹, using *Synechococcus* sp. WH7803 from Warwick. Plaque diameters after 10 days ranged from 0.5-1.5 mm. Six plaques were subsequently picked and resuspended in ASW. Plaque isolation, using *Synechococcus* sp. WH8018 from Warwick, attempted on seawater from Hydrostation S resulted in no plaques. However, seawater from coastal Bermuda gave seven plaques from 100 µl, using *Synechococcus* sp. WH8018, mainly 0.5 mm

in diameter after one week; however, there were two plaques 12 mm in diameter, which were subsequently picked, along with three small plaques.

All the plaques which were picked were purified by two further rounds of plaque purification (2.6.1.1), using the strain on which they were isolated, before using a final plaque to obtain a lysate from a liquid culture. This lysate was then filtered through a 0.22 μm pore-size membrane and stored as a cyanophage stock at 4°C in the dark.

3.3 Cyanophage Characterisation

3.3.1 Morphology

Cyanophages which had been isolated and plaque-purified were characterised morphologically by transmission electron microscopy (TEM) (2.6.2). If the cyanophage titre from a lysate was not high enough for TEM characterisation then cyanophages were concentrated by PEG precipitation, as for a DNA preparation (2.8.1). Optimisation of the preparation of specimens was necessary to get sufficient cyanophages, yet with as few salt crystals as possible. This was achieved by varying: (a) the length of time for which the specimen was allowed to dry on the TEM grid; (b) the amount of washing of the specimen; and (c) the length of time for staining. The optimum conditions are described in section 2.6.2.2.

Cyanophages were classified according to their morphological structure (see Fig. 1.2) Cyanophages isolated from Hydrostation S, on *Synechococcus* sp. WH7803, were classified as Myoviridae isolates and named cyanophage strains S-BM3 and S-BM6. S-BM3 is shown in Figure 3.1a. "S" represents the host genus (*Synechococcus*); "B" represents the region of isolation (Bermuda); and "M" represents the family (Myoviridae) (see Wilson *et al.*, 1993). A cyanophage isolated from coastal Bermuda on *Synechococcus* sp. WH7803 was classified as a Podoviridae isolate and named

cyanophage strain S-BP3 (Fig. 3.1b). Two cyanophages isolated from coastal Bermuda on *Synechococcus* sp. WH8018, which formed small plaques, were classified as Myoviridae isolates and named cyanophage strains S-BM4 and S-BM5. S-BM4 is shown in Figure 3.1c. The two cyanophages isolated from coastal Bermuda on *Synechococcus* sp. WH8018, which formed very large plaques, were both Podoviridae isolates and named cyanophage strains S-BP1 and S-BP2. S-BP1 is shown in Figure 3.1d. Cyanophage dimensions were calculated from the TEMs, calibrating with 50 nm plastic beads, and are summarised in Table 3.1, together with genome sizes, which were calculated after restriction digestion of cyanophage DNA (see 3.3.2). Table 3.1 shows that the myoviruses have similar size genomes, but which are larger than those of the podoviruses.

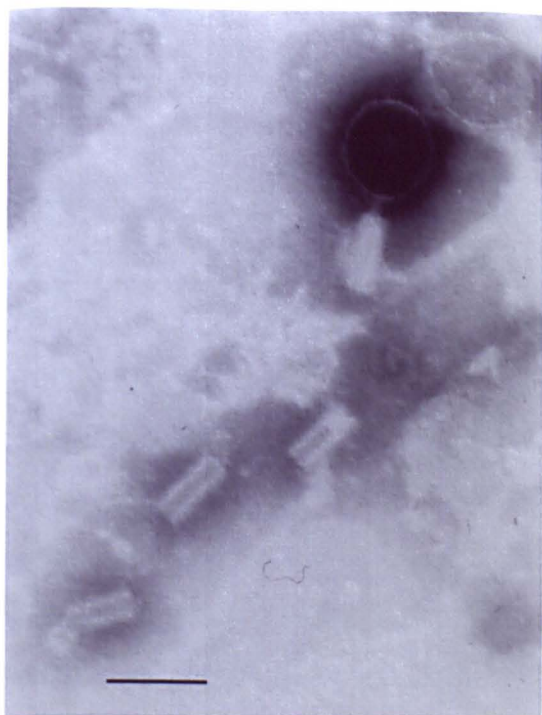
Table 3.1 Cyanophage dimensions. Cyanophages were isolated on *Synechococcus* sp. WH7803, from Hydrostation S, Sargasso Sea (cyanophage strains S-BM3 and S-BM6), and coastal Bermuda (cyanophage strains S-BM1, S-BM4, S-BM5, S-BP1, S-BP2 and S-BP3), according to TEM analysis. Cyanophage strain S-BM1 was isolated and characterised by Wilson *et al.* (1993). T4, a phage of *Escherichia coli*, is shown as a comparison (see Birge, 1994). Phage dimensions are given as the mean \pm standard deviation ($n = 3$). Genome sizes were calculated by the summation of fragments from a single restriction digest (2.8.3), comparing with a 1 kb ladder marker.

Phage	Head	Tail length (nm)		Tail width (nm)	Genome size (kb)
	diameter (nm)	Extended	Contracted		
S-BM1	88		57	31	144
S-BM3	113 \pm 5.8	189 \pm 41.2	84 \pm 5.2	33 \pm 2.3	134
S-BM4	119 \pm 9.9	228 \pm 40.1	82 \pm 9.3	36 \pm 0.0	158
S-BM6	118 \pm 8.7	217 \pm 30.6	79 \pm 9.5	26 \pm 4.0	
S-BP1	127 \pm 8.1	-	-		44
S-BP2	83 \pm 5.8	-	-		47
S-BP3	70 \pm 4.0	-	-		45
T4	95	113		20	168

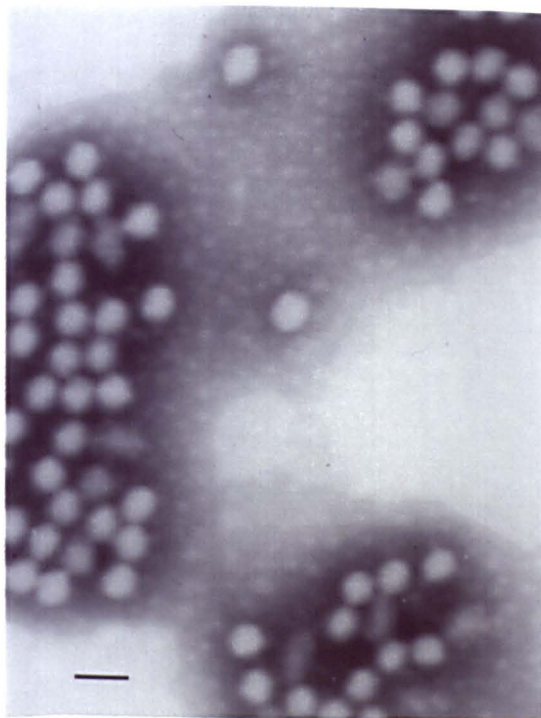
3.3.2 DNA restriction and Southern hybridisation

From each of the cyanophages which had been plaque purified (3.2), DNA was prepared (2.8.1.1) and analysed by restriction endonuclease digestion (2.8.3; 2.8.4), to confirm their individuality, and subsequently by Southern hybridisation (2.8.8b), using a 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2 as a probe (Fig. 3.2). This probe had previously been found to cross-hybridise with DNA from other

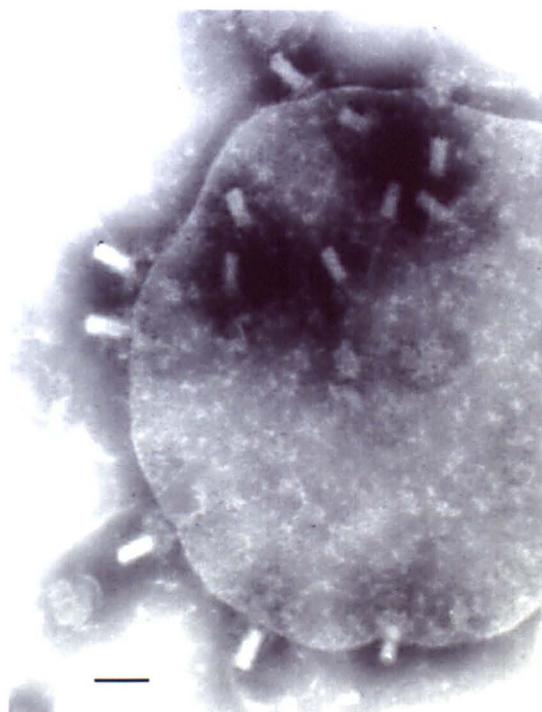
(a)



(b)



(c)



(d)

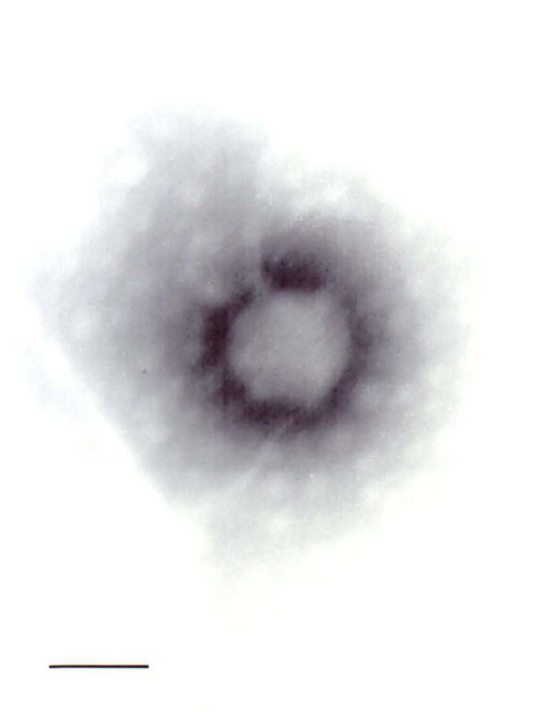


Figure 3.1 Transmission electron micrographs of marine cyanophages. Cyanophage strain S-BM3 was isolated from the Sargasso Sea; cyanophage strains S-BM4, S-BP1 and S-BP3 were isolated from coastal Bermuda. Cyanophage strains S-BM3 and S-BP3 were isolated on *Synechococcus* sp. WH7803; cyanophage strains S-BM4 and S-BP1 were isolated on *Synechococcus* sp. WH8108. Cyanophage strains S-BM3 and S-BM4 belong to family Myoviridae; cyanophage strains S-BP1 and S-BP3 belong to family Podoviridae. Cyanophage strains are: (a) S-BM3; (b) S-BP3; (c) S-BM4; (d) S-BP1. The bar represents 100 nm.

marine cyanophages (Wilson, 1994). The cyanopodovirus, $\Phi 12$, kindly donated by John Waterbury (Waterbury and Valois, 1993), was plaque-purified and similarly analysed. This cyanophage, like S-BP1 and S-BP2, also produced large plaques.

Restriction patterns (Fig. 3.2a) confirm the individuality of cyanophage strains S-BM3, S-BM4, S-BM5, S-BP1, S-BP2 and S-BP3, although cyanophage strains S-BM4 and S-BM5 differ by only three bands. These cyanophages are all different from cyanophage strain S-BM1, which had been previously isolated from coastal Bermuda on *Synechococcus* sp. WH7803 (Wilson *et al.*, 1993). Southern hybridisation shows cross-hybridisation of the probe to the cyanomyoviruses from coastal Bermuda and Hydrostation S, isolated on *Synechococcus* sp. WH7803 (cyanophage strains S-BM1 and S-BM3 respectively), and to the two cyanomyoviruses from coastal Bermuda, isolated on *Synechococcus* sp. WH8018 (cyanophage strains S-BM4 and S-BM5). However, none of the cyanopodoviruses (cyanophage strains S-BP1, S-BP2, S-BP3 and $\Phi 12$) showed cross-hybridisation.

3.3.3 Host range

Host range studies were performed on cyanophages isolated from Hydrostation S and coastal Bermuda. Plaque formation (2.6.1.1) and well clearance (2.6.1.2), using *Synechococcus* sp. strains WH7803, WH8018 and WH8103, were used to determine the host range of the cyanophages (Table 3.2). Formation of plaques, or cleared wells, indicated that the cyanophage infected that host strain. Plates and wells were left for 2-3 weeks before a definite negative result was given. *Synechococcus* sp. WH8103 could not be grown on plates, so the well clearance method was adopted for this strain. Table 3.2 shows that there appears to be a great diversity in host range even amongst cyanophages isolated from the same place. Some cyanophages infect more than one of these strains, whilst others infect only a single strain.

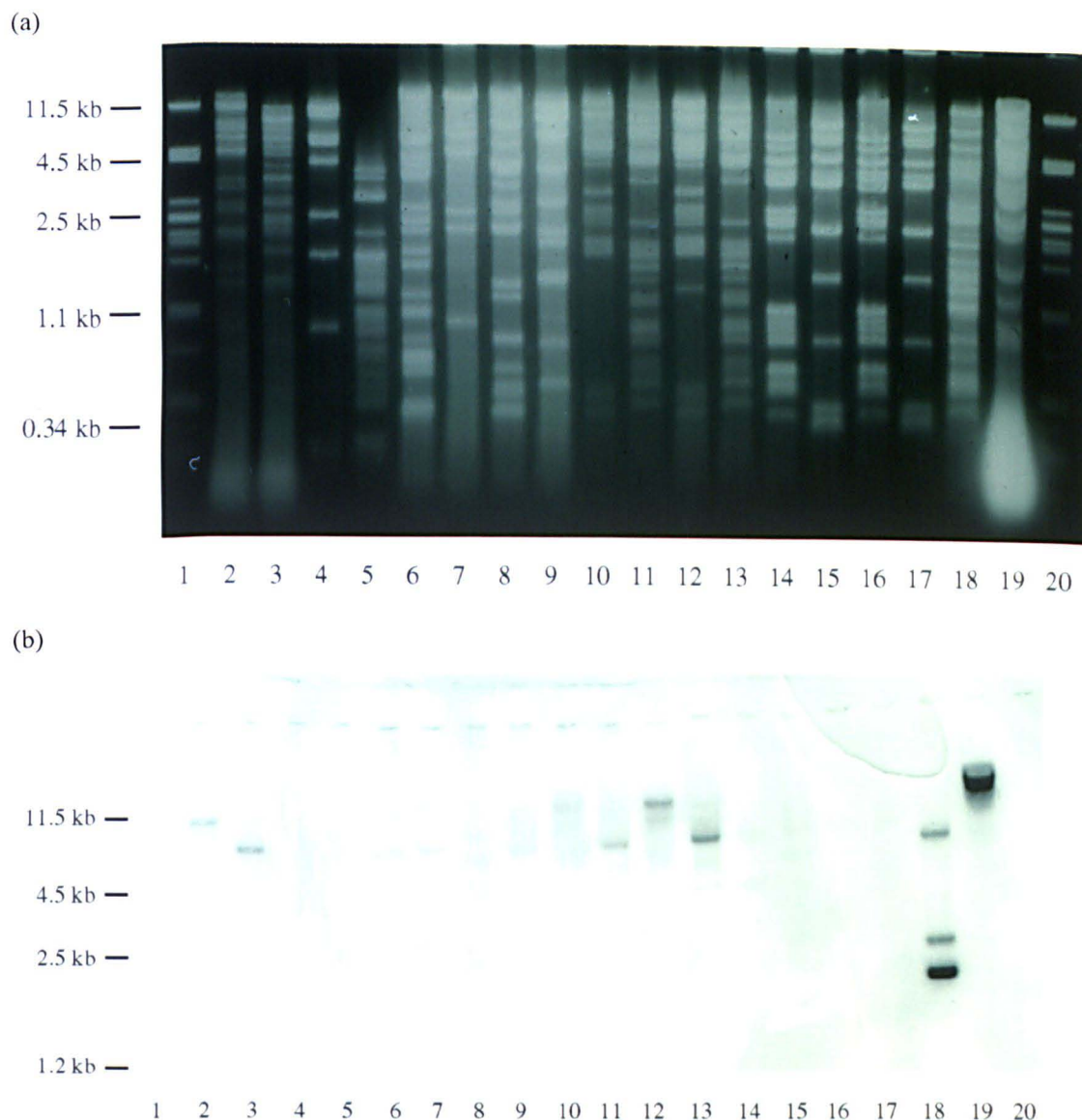


Figure 3.2 Southern analysis of cyanophage digests, using a 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2 as a probe.

(a) Restriction endonuclease digestion of DNA from cyanophages isolated from the Sargasso Sea (cyanophage strain S-BM3) and coastal Bermuda (cyanophage strains S-BM1, S-BM4, S-BM5, S-BP1, S-BP2 and S-BP3). Cyanophage strain S-BnM1 was isolated from seawater off the coast of Bergen, Norway, and together with cyanophage strain S-BM1, were isolated by Wilson *et al.* (1993). Cyanophage strain Φ 12 is a cyanopodovirus from the Gulf Stream off the coast of Virginia (Waterbury and Valois, 1993). Tracks 1 and 20 contain the marker, phage λ cut with *Pst*I. Other tracks contain the following restrictions (cyanophage strain, enzyme): (2) S-BM3, *Bam*HI; (3) S-BM3, *Eco*RI; (4) S-BP3, *Bam*HI; (5) S-BP3, *Eco*RI; (6) S-BP1, *Bam*HI; (7) S-BP1, *Eco*RI; (8) S-BP2, *Bam*HI; (9) S-BP2, *Eco*RI; (10) S-BM4, *Bam*HI; (11) S-BM4, *Eco*RI; (12) S-BM5, *Bam*HI; (13) S-BM5, *Eco*RI; (14) Φ 12, *Bam*HI; (15) Φ 12, *Eco*RI; (16) Φ 12, *Bam*HI; (17) Φ 12, *Eco*RI; (18) S-BnM1, *Pst*I/*Eco*RI; (19) S-BM1, *Bam*HI.

(b) Corresponding Southern hybridisation filter probed with a 1 kb *Bam*HI/*Eco*RI fragment, encoding a T4 g20 homologue, from cyanophage strain S-PM2. Hybridisation conditions were 55°C overnight with 5x SSC, 0.02% (w/v) SDS, followed by a 10 min wash at room temperature and two 15 min washes at 55°C, each with 2x SSC, 0.1% (w/v) SDS.

Table 3.2 Host range of cyanophages which infect *Synechococcus* sp. strains. Cyanophages were isolated from the Sargasso Sea (cyanophage strains S-BM3 and S-BM6) and coastal Bermuda (cyanophage strains S-BM1, S-BM4, S-BM5, S-BP1, S-BP2 and S-BP3). Cyanophage strain S-BM1 was isolated by Wilson *et al.* (1993). "+" indicates that the cyanophage infects this host, "-" indicates that it does not infect it; * indicates that the cyanophage was isolated on this strain; a space indicates that this host was not tested with this cyanophage.

Cyanophage strain	Host strain		
	WH7803	WH8018	WH8103
S-BM1	+	+	-
S-BM3	+	-	+
S-BM4	+	+	-
S-BM5	-	+	
S-BM6	+		
S-BP1	-	+	-
S-BP2	-	+	-
S-BP3	+	-	-

3.4 Attempted Isolation of Viruses Infecting Marine Heterotrophic Bacteria

3.4.1 Isolation of marine heterotrophic bacteria

Seawater samples collected from Plymouth, *ca.* four miles offshore, and from Hydrostation S, Sargasso Sea, were used to isolate marine heterotrophic bacteria (2.7.1). Bacterial titres were *ca.* 10^3 CFU ml⁻¹, and *ca.* 10^4 CFU ml⁻¹ in the Plymouth and Hydrostation S samples respectively. Four different colony morphotypes from the Plymouth sample were purified (2.7.1) and named PL5, PL6, PL7 and PL8. Four different colony morphotypes from Hydrostation S were also purified and named HS1, HS2, HS3 and HS4. PL5, PL6, HS1, HS2 and HS3 grew up overnight, while PL7, PL8 and HS4 were more slow-growing, taking about a week to form 1mm-diameter colonies. It was noticed that PL5 and PL6 released a very foul, sewage-like smell.

3.4.2 Attempted isolation of bacteriophages

Bacteriophage isolation was attempted by plaque formation (2.7.2), taking each bacterium in its mid-exponential phase, according to their growth curves (data not shown). Phage isolation was attempted on the Plymouth bacteria. The seawater collected from Plymouth, from which the bacteria had been isolated, was used as a potential source of phages, using techniques previously described (Frank and Moebus, 1987). Phage isolation was also attempted on the Sargasso Sea bacteria. The seawater collected from Hydrostation S (from which the bacteria had been isolated), coastal Bermuda and coastal Plymouth were similarly used as putative sources of phages. Whilst good lawns were obtained for each of the bacteria tested, no plaques were obtained on any of the lawns with any of the water samples, whether filtered or unfiltered.

A selection of three bacteriophages infecting marine heterotrophic bacteria was kindly donated by Karlheinz Moebus, representing each of the three families to which marine cyanophages belong. Phage strain H2/1 belongs to the family Myoviridae and infects bacterial strain H2; phage strain H4/4 belongs to the family Podoviridae and infects bacterial strain H4; and phage strain H54/1 belongs to the family Siphoviridae and infects bacterial strain H54 (Frank and Moebus, 1987). All the phages were isolated from the North Sea. These phages were plaque-purified (2.7.2) and DNA preparations made from plate lysates (2.8.1.2). Their DNA, along with T4 DNA, was then restricted (2.8.3; 2.8.4) and analysed by Southern hybridisation (2.8.8b), using the 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2 as a probe (Fig. 3.3). Figure 3.3 shows that the cyanophage probe cross-hybridised with DNA from cyanophage strain S-BnM1 (as expected), but not with DNA from T4, nor from the phages of marine heterotrophic bacteria.

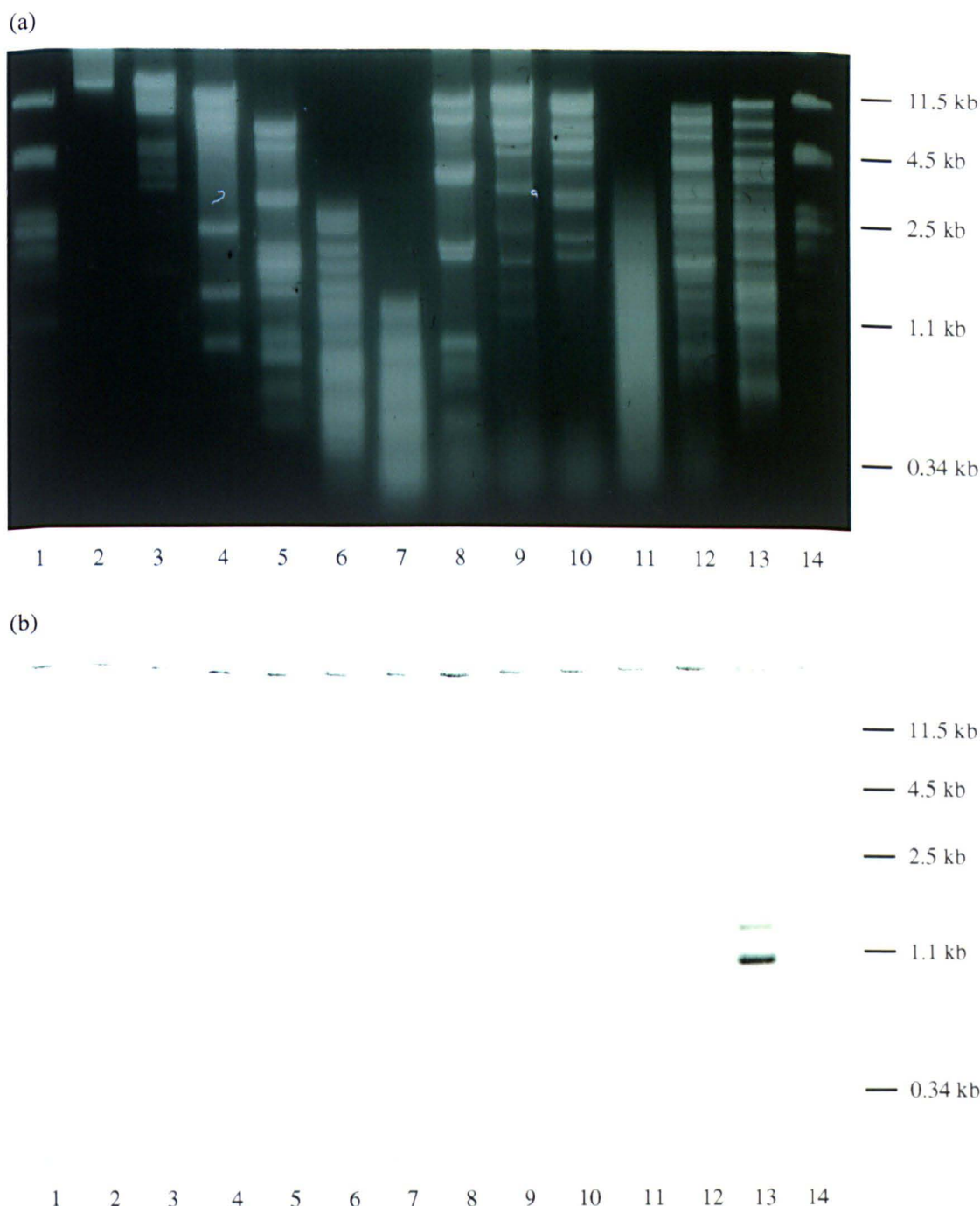


Figure 3.3 Southern analysis of bacteriophage DNA, using the T4 g20 homologue from cyanophage strain S-PM2 as a probe.

(a) Restriction digests of DNA from bacteriophages infecting marine heterotrophic bacteria (phage strains H2/1, H4/4 and H54/1; Frank and Moebus, 1987), the coliphage T4 and cyanophage strain S-BnM1. Tracks contain the following (phage, enzyme): (1) λ , *Pst*I; (2) T4, *Eco*RI; (3) T4, *Eco*RV; (4) H2/1, *Eco*RI; (5) H2/1, *Eco*RV; (6) H2/1, *Sau*3A; (7) H4/4, *Alu*I; (8) H4/4, *Hind*III; (9) H4/4, *Xba*I; (10) H54/1, *Eco*RI; (11) H54/1, *Eco*RV; (12) H54/1, *Hind*III; (13) S-BnM1, *Pst*I/*Eco*RI; (14) λ , *Pst*I.

(b) Corresponding Southern hybridisation filter probed with a 1 kb *Bam*HI/*Eco*RI fragment, encoding a T4 g20 homologue, from cyanophage strain S-PM2. Hybridisation conditions were 51°C for 6 h with 5x SSC, 0.02% (w/v) SDS, followed by a 10 min wash at room temperature and a 15 min wash at 51°C, each with 2x SSC, 0.1% (w/v) SDS.

3.5 Discussion

3.5.1 Isolation of cyanophages

A limitation of using microbiological techniques to isolate cyanophages is the host strain specificity of the cyanophages. There may always be cyanophages which are not detected simply because they will not infect the host strains used. For this reason the results will always be skewed in analysing environmental samples, and the cyanophages isolated will not necessarily be representative of the natural population; they will not necessarily be the most abundant cyanophage strains. For example, Waterbury and Valois (1993) observed that *Synechococcus* sp. strains WH7803 and WH8018 were more susceptible to infection by cyanophages than were other host strains. Furthermore, Suttle and Chan (1994) observed highest cyanophage counts in marine waters when they used *Synechococcus* sp. strains WH7803 and SYN48.

Whilst the plaque formation method was preferentially used for isolating cyanophages (3.2), it should be noted that this method involves a brief treatment of the cyanophages at 42°C before being poured, with the molten agar, on to a solid medium plate (2.6.1.1). This change in temperature may possibly have had a deleterious effect on some cyanophages, especially those from colder oceanographic regions, and may have resulted in a loss of infectivity. Whilst Hidaka (1972) observed that many marine phages did not lose infectivity at 50°C, many phages isolated from cold seawater have a decreased plaquing efficiency, even when incubated at 25°C (Moebus, 1983).

Whilst no *Synechococcus* spp. counts were made on most of the seawater samples used for plaque isolation, it is surprising that most of the samples resulted in no plaque formation, since cyanophages which infect *Synechococcus* sp. WH7803 have been isolated from many oceanographic regions (Wilson, 1994). However, whilst Suttle and Chan (1993) detected cyanophages from every water sample tested (in warmer waters of the west Atlantic), Wilson *et al.* (1993) could not detect

cyanophages in every sample from the Plymouth coast. It is possible that the *Synechococcus* spp. concentrations in the samples in this study were too low to produce detectable numbers of cyanophages. Suttle and Chan (1994) observed that a threshold of *ca.* 10^3 *Synechococcus* spp. cells ml⁻¹ was necessary for cyanophage concentrations to increase from 10^2 to 10^5 infective units ml⁻¹.

Another possibility for the inability to isolate cyanophages is the degradation of cyanophages during the time between collection and plaque isolation (3.2). Most of these samples were refrigerated in the dark after collection, but then subjected to air transport for several hours in the dark at room temperature, before being filtered. The long time delay between collection and filtration could have resulted in cyanophage degradation. Particles larger than 0.22 μm are well known for causing considerable virus decay in seawater (Suttle and Chen, 1992 and Noble and Fuhrman, 1997). The apparent decrease in titre in the Hydrostation S sample over the four-week period, between attempted cyanophage isolation in Bermuda and then in Warwick, may be a result of degradation from heat-labile dissolved substances, such as proteases (Fuhrman and Noble, 1997).

Concentrations of cyanophages which infect *Synechococcus* sp. WH7803, at Hydrostation S were about an order of magnitude less than the total *Synechococcus* spp. concentration (3.2). Higher cyanophage concentrations would not be expected, according to Suttle and Chan (1994), since the host concentration (6×10^2 ml⁻¹) had not reached the threshold of 10^3 ml⁻¹ which is necessary for efficient cyanophage propagation (Suttle and Chan, 1994).

The sample from coastal Bermuda, though collected two weeks after the Hydrostation S sample, had a titre approximately an order of magnitude higher than the Hydrostation S sample (3.2). This is despite the fact that the Bermuda sample was subjected to air transport before attempting plaque isolation, unlike the Hydrostation S sample. This would be in accordance with previous research which has shown higher cyanophage concentrations in coastal, compared with offshore, regions (Suttle and Chan, 1994). However, it should be noted that the Hydrostation S sample was

taken before Hurricane Felix hit the area, yet the coastal sample was taken about 10 days after the hurricane. The hurricane, stirring up deeper, nutrient-rich waters, may have resulted in increased *Synechococcus* spp. growth (Waterbury *et al.*, 1986) and consequently in increased cyanophage concentrations.

3.5.2 Cyanophage characterisation

3.5.2.1 Morphology

Classification of cyanophages by TEM (3.3.1) to the family Podoviridae was on the basis of the presence of heads and absence of any visible tails, but the possession of a small stump. Heads were often observed in clusters (Fig. 3.1). Classification as Myoviridae isolates was on the basis of a contractile tail; however, the uncontracted form was rarely observed, and often a long, thin core was observed, protruding from a contracted tail sheath. Such an observation was also made by Suttle and Chan (1993). The presence of such cores was interpreted as evidence of a formerly uncontracted tail, and the cyanophage was thus assigned to the family Myoviridae.

Morphological characterisation of the cyanophages isolated from Hydrostation S and Bermuda (3.3.1) showed that four out of seven isolates were cyanomyoviruses, while the remaining three were cyanopodoviruses. This result is in accordance with previous research, such as Waterbury and Valois (1993), who isolated many cyanomyoviruses, but also some cyanopodoviruses. In addition, all the cyanophages isolated by Wilson (1994) belong to the family Myoviridae, although some were initially misclassified as cyanosiphoviruses and cyanopodoviruses. No cyanosiphoviruses were found. Suttle and Chan (1993) isolated a single cyanosiphovirus, but this only infected a phycoerythrin-absent coastal *Synechococcus* sp. strain; all their cyanophages which infected phycoerythrin-containing *Synechococcus* spp. strains were cyanomyoviruses. Waterbury and Valois (1993) reported the isolation of cyanosiphoviruses, but the majority of isolates were cyanomyoviruses.

TEM analysis showed little morphological variation within a cyanophage family, according to the cyanophage dimensions, although cyanophage strain S-BM1 is somewhat smaller than the other cyanomyoviruses (Table 3.1). In contrast, Waterbury and Valois (1993) observed greater morphological diversity amongst cyanophages. Cyanophages isolated in this study are all within the range of sizes which have already been observed for marine cyanophages (Suttle and Chan, 1993; Waterbury and Valois, 1993; Ohki and Fujita, 1996), freshwater cyanophages (*e.g.* Hu *et al.*, 1981; Kim and Choi, 1994) and marine bacteriophages in the corresponding families (Hidaka and Fujimura, 1971; Frank and Moebus, 1987; Kellogg *et al.*, 1995), and are similar in size to T4.

Calculations of genome sizes (Table 3.1) show that all the cyanopodoviruses appear to have considerably smaller genomes than the cyanomyoviruses, as might be expected for cyanophages without contractile tails, since they would have fewer capsid proteins to encode. All the cyanophages within the same family appear to have similar sized genomes, suggesting a standardisation of genetic content for cyanophages in the same family, as is commonly observed amongst phages and other viruses (for review see Dimmock and Primrose, 1994). In addition, the Myoviridae isolates have similar genome sizes to T4, also a myovirus. However, these cyanomyoviruses have genomes which are 2-3 times larger than myoviruses which infect the marine bacterium *Vibrio parahaemolyticus* (Kellogg *et al.*, 1995), and have genomes which are a similar size to those of some viruses of eukaryotic algae (*e.g.* Ivey *et al.*, 1996). Cyanomyovirus genome sizes were slightly larger than those previously reported for marine cyanomyoviruses (Wilson *et al.*, 1993). However, the podoviruses in this study had considerably smaller genomes than the putative podovirus isolated by Wilson (cyanophage strain S-RSP1; Wilson, 1994), suggesting that cyanophage strain S-RSP1 may have been misclassified.

3.5.2.2 DNA restriction and Southern hybridisation

Restriction patterns of cyanophage DNA (Fig. 3.2a) show that all the cyanophages isolated are different from cyanophage strain S-BM1, which was isolated from coastal Bermuda three years earlier, 20 miles away from the more recent site of isolation. Given the diversity of cyanophages within an individual seawater sample (Waterbury and Valois, 1993; Chapter 6 this study), and the 20-mile separation, it is not surprising that cyanophage strain S-BM1 was not isolated again three years later.

Southern hybridisation analysis (Fig. 3.2b) shows homology between the fragment from cyanophage strain S-PM2 and all of the cyanomyovirus isolates. There appears to be no homology between the fragment from cyanophage strain S-PM2 and any of the cyanopodovirus isolates. This suggests that the cyanophage T4 gp20 homologue is conserved only in the family Myoviridae, and not Podoviridae. T4 itself belongs to the family Myoviridae, and is 53% identical to the cyanophage probe. However, T4 DNA did not cross-hybridise with the cyanophage probe, even under less stringent conditions (Fig. 3.3b). Hence all that can be concluded is that the Podoviridae isolates are not as similar as the Myoviridae isolates to the cyanophage T4 gp20 homologue. However, it is unlikely that cyanopodoviruses would possess this T4 gp20 homologue, since T4 gp20 has shown no significant similarity with any podoviruses on the computer database (Wilson, 1994). Whilst no cyanosiphoviruses could be isolated and probed for homology, it is also unlikely that they would possess the homologue, since no siphoviruses on the database showed significant similarity to T4 gp20 (Wilson, 1994).

3.5.2.3 Host range

Host range analysis (Table 3.2) revealed a wide range of infectivity, even using just three host strains. Some cyanophages will only infect one of the strains, whereas others infect more. Furthermore, the combination of host strains infected by a cyanophage is diverse. Such variations have been observed previously (Suttle and

Chan, 1993; Waterbury and Valois, 1993). Using a fourth host, additional to those in this study, *Synechococcus* sp. WH8012, Wilson (1994) showed that several cyanophages infected three host strains.

There appears to be little correlation between host range and place of isolation (Table 2.3), as observed also by Suttle and Chan (1993). However, cyanophage strain S-BM3, isolated from the Sargasso Sea, is the only one of the cyanophages which infected *Synechococcus* sp. WH8103, suggesting that this host is not as susceptible to infection by cyanophages isolated from coastal regions. All of the cyanophages isolated by Wilson (1994) were isolated from coastal regions, and none would infect *Synechococcus* sp. WH8103. *Synechococcus* sp. WH8103 was isolated from the open ocean (Waterbury *et al.*, 1986), suggesting a link between this oceanic cyanophage (cyanophage strain S-BM3) infecting an oceanic host strain. However, *Synechococcus* sp. WH7803 was also isolated from the open ocean (Waterbury *et al.*, 1986), and yet was infected both by oceanic and coastal cyanophages (Table 3.2). *Synechococcus* sp. WH8018 was isolated from the coast and was not infected by the one oceanic cyanophage. However, Waterbury and Valois (1993) have isolated two cyanophages from the open ocean which did infect *Synechococcus* sp. WH8018. Therefore, the place of isolation of a host strain would appear to have little bearing on its range of susceptibility to infection, although there is clearly a difference in susceptibility to infection between host strains; *Synechococcus* sp. WH7803 appears more susceptible than *Synechococcus* sp. WH8103, as also observed by Waterbury and Valois (1993).

Host range does not appear to be correlated with homology to the fragment from cyanophage strain S-PM2 (Table 3.2 and Fig. 3.2b). Both cyanophage strains S-BM3 and S-BM5 cross-hybridised, and yet cyanophage strain S-BM3 infected *Synechococcus* sp. strains WH7803 and WH8103, but not *Synechococcus* sp. WH8018. Conversely, cyanophage strain S-BM5 infected *Synechococcus* sp. WH8018, but not *Synechococcus* sp. strains WH7803 or WH8103. Homology between cyanophages and the fragment from cyanophage strain S-PM2 appears to be

solely on the basis of being in the family Myoviridae. Only cyanomyovirus isolates cross-hybridised to the fragment, and all cyanomyovirus isolates cross-hybridised.

Comparing host range analysis with restriction patterns (Table 3.2 and Fig. 3.2a) it is interesting to note that cyanophage strains S-BM4 and S-BM5 appear to differ only in the existence of a *Bam*HI site to exchange an 8 kb fragment in cyanophage strain S-BM4 for a 7 kb and 1 kb fragment in cyanophage strain S-BM5, yet this difference also accompanies a difference in host range. Unlike cyanophage strain S-BM4, cyanophage strain S-BM5 was unable to infect *Synechococcus* sp. WH7803. This would suggest that this *Bam*HI site is found in a variable region responsible for altering host range. It may be speculated that this region encodes a homologue of the tail fibre protein, T4 gp37, which determines host specificity of T4 (see review by Heller, 1992).

3.5.3 Attempted isolation of viruses infecting marine heterotrophic bacteria

When isolating marine heterotrophic bacteria (3.4.1) it was noticed that the bacterial titres in the seawater were only 10^3 - 10^4 CFU ml⁻¹. This is vastly lower than total bacterial counts, obtained by DAPI staining and microscopy, which are typically, and fairly constantly, *ca.* 10^6 ml⁻¹ (*e.g.* Proctor and Fuhrman, 1992; Cochlan *et al.*, 1993). Low counts may be due to the method of isolation, which will only select for those bacteria capable of growing on the given compounds and in the given conditions (2.7.1). This may well be a tiny fraction of the total marine bacterial population (for review see Fuhrman *et al.*, 1994). Hence, in isolating any bacteria, it is unlikely that they will be representative of the actual population, indeed the foul smell and rapid growth rate of the bacterial strains PL5 and PL6 suggest that they may be contaminants from a sewage outfall.

The fact that no bacteriophages were found to infect any of the bacteria isolated (3.4.2) may be a result of the biased selection, perhaps isolating bacteria which are relatively less abundant, and which would therefore have a lower associated phage population, perhaps too low for detection. It may also be that the bacteria isolated were relatively resistant to infection from phages in the same water sample (an observation from Waterbury and Valois, 1993), due to repeated exposure to them and natural selection. However, this would not explain why Sargasso Sea bacteria were not infected when treated with seawater from another region. In this case it is possible that the bacteria were localised to that specific region of isolation. Indeed, Moebus and Nattkemper (1981; cited by Borsheim, 1993) observed that bacteria isolated from west of the mid-Atlantic ridge were rarely infected by phages isolated from east of the ridge. It is quite possible that there were just too few phages to be detected, since concentrations of specific phages are generally low (*e.g.* Moebus, 1991; 1992b). It may be necessary to concentrate phages, by enrichment or tangential flow filtration, since phages are notoriously difficult to isolate directly from seawater samples (*e.g.* Hidaka and Fujimura, 1971; Moebus, 1980).

The results of the attempted Southern hybridisation of the fragment from cyanophage strain S-PM2 to the phages of heterotrophic bacteria (Fig. 3.3) were as expected, if the fragment is conserved only amongst cyanophages. Given that the fragment did not cross-hybridise with the cyanopodoviruses (Fig. 3.2), it is not surprising that it did not cross-hybridise with the Podoviridae isolate H4/4. The fact that it did not cross-hybridise with the Myoviridae isolate H2/1 suggests that the fragment is indeed conserved only in cyanophage myoviruses, to the point of detection by Southern hybridisation under the conditions used (Fig. 3.3). It should be noted that T4 did not cross-hybridise either, so whilst these phages of marine heterotrophic bacteria are not sufficiently similar to the T4 g20 homologue in cyanophages for Southern hybridisation, they (particularly the myovirus) could still possess their own T4 g20 homologue.

Chapter 4:

Molecular Characterisation of T4 Gene 20 Homologues in Three Cyanophages

4.1 Introduction

Previous analysis of marine cyanophages has concentrated on abundance, morphological characterisation by TEM, growth kinetics and host range (Suttle and Chan, 1993; Waterbury and Valois, 1993; Suttle and Chan, 1994). Relatively little research has been into their molecular characterisation to date (Wilson *et al.*, 1993; Wilson, 1994; Ohki and Fujita, 1996), and yet molecular characterisation is able to give a much greater resolution in population studies, and has the potential for the development of analytical methods with either a very broad or very specific range for detection of cyanophages. A major limitation with non-molecular analysis of cyanophages is their initial isolation. Many cyanophages may be very specific to a single, or limited number of host strains, as revealed in Chapter 3 of this study (also Waterbury and Valois, 1993). Yet cyanophages are traditionally isolated by infection of a limited selection of host strains (Suttle and Chan, 1993; Wilson *et al.*, 1993). Hence, it is quite possible that a large proportion of cyanophage strains are not isolated by these classical microbiological techniques. Molecular techniques, specifically DNA probes, have the potential to detect a broad range of cyanophages, crossing the barrier of host strain specificity, and yet remain specific to cyanophages as a virus group (Chen and Suttle, 1995a). In addition, subtle changes in the cyanophage DNA may result in a change in host strain specificity and yet no alteration in morphology (Chapter 3, this study), or perhaps no detectable phenotypic change. Hence DNA analysis, as a more sensitive technique, would detect such diversity which other traditional methods, such as TEM, would not detect.

This chapter describes the design of molecular probes for the subsequent analysis of cyanophage populations, continuing work begun by Wilson (1994). The target for the design of probes is a DNA region previously found to be conserved amongst a selection of cyanophages.

Having isolated several cyanophages from different oceanographic regions which showed homology with each other by Southern hybridisation, Wilson isolated and

cloned a separate DNA fragment, containing part of the homologous region, from each of three cyanophages, and gained partial sequence data (Wilson, 1994). The cyanophage strains were isolated from Plymouth, UK (S-PM2, formerly named S-PS1), Woods Hole, USA (S-WHM1) and Bergen, Norway (S-BnM1, formerly named S-BnS1). Partial sequence data for cyanophage strains S-PM2 and S-WHM1 revealed limited homology (46% and 41% similarity at the peptide level) to gene 20 from the coliphage T4, which encodes a capsid assembly protein (Marusich and Mesyanzhinov, 1989). The gene product, T4 gp20, is described in Chapter 3 of this study. Such a DNA region, apparently conserved amongst cyanophages, showed potential for use as a target for the development of cyanophage-specific DNA probes, in the form of PCR primers, and subsequent molecular analysis of natural cyanophage populations. The results of Chapter 3 of this study suggest that this DNA region is conserved in cyanomyoviruses, but not cyanopodoviruses. However, cyanomyoviruses appear to constitute the majority of marine cyanophages (Waterbury and Valois, 1993).

4.2 Aims

The aim of the research described in this chapter was to complete the sequence data for all three DNA fragments, from cyanophage strains S-PM2, S-WHM1 and S-BnM1, and to isolate further DNA fragments, if necessary, to complete the sequence of the entire genes. Then PCR primers would be designed from regions conserved amongst the cyanophages, in order to amplify cyanophage, but not T4, DNA. The majority of the contents of this chapter are the basis of a manuscript submitted to Applied and Environmental Microbiology (Fuller *et al.*, 1998. In press).

4.3 Isolation of DNA Fragments Containing the T4 Gene 20 Homologues From Three Cyanophages

4.3.1 Isolation of the T4 gene 20 homologue from cyanophage strain S-PM2

A 1 kb *Bam*HI/*Eco*RI fragment, containing part of the T4 g20 homologue from cyanophage strain S-PM2, had been cloned into pUC19 to generate pWHW02 (Wilson, 1994). Initially the ends of the pWHW02 1 kb insert were sequenced following ligation into both M13mp18 and M13mp19 to obtain sequence data for both orientations (2.8.9). In addition, both strands of the pWHW02 insert were fully sequenced by random subcloning into *Sma*I-cut M13mp18, using the self-ligation and sonication method (2.8.9.1-2.8.9.5). Comparison of the sequence data from the 1 kb fragment with T4 g20 suggested that there could be *ca.* 400 bp of open reading frame upstream of the 5' *Eco*RI end of the fragment, and *ca.* 150 bp downstream of the 3' *Bam*HI end. Hence, additional DNA fragments had to be cloned from cyanophage strain S-PM2 to complete the sequence of the gene. For a schematic diagram of the T4 g20 homologue region, see Figure 4.6a.

To obtain sequence data upstream of the 5' *Eco*RI end of the 1 kb fragment the plasmid pWHW03 was used, which contained a 20 kb *Bam*HI insert, containing the upstream region of the T4 g20 homologue from cyanophage strain S-PM2. pWHW03 was restricted with a range of enzymes (2.8.3) and resulting digests were analysed by Southern hybridisation (2.8.8a) to identify a smaller fragment containing the upstream region of the T4 g20 homologue from cyanophage strain S-PM2 (Fig. 4.1).

Comparison of the resulting Southern blot with the restriction digest (Fig. 4.1) revealed that restricting pWHW03 with *Cla*I generated a 4.5 kb and a 1 kb fragment which both cross-hybridised with the probe. Analysis of a restriction map of the known sequence data (Fig. 4.6a) revealed that the 1 kb *Cla*I fragment contained the 5'

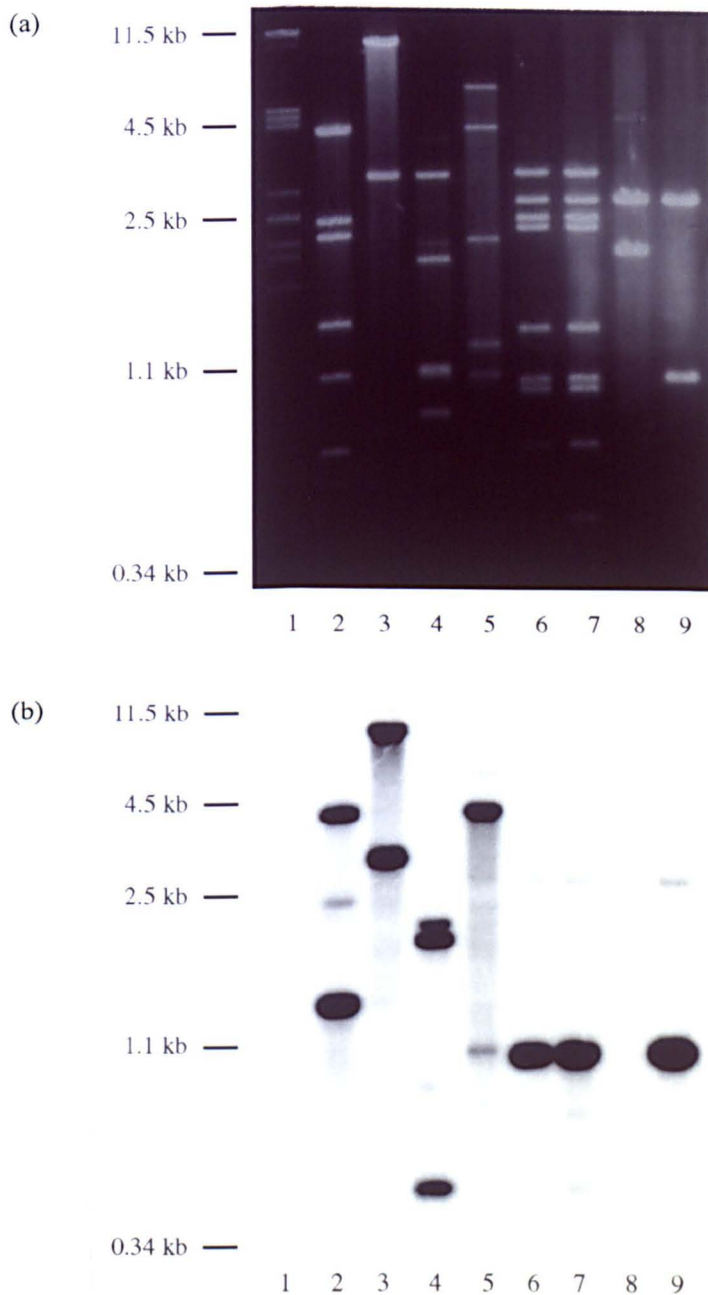


Figure 4.1 Southern analysis of pWHW03 digests using the pWHW02 probe.

(a) Restriction endonuclease digestion of pWHW03 (which contains a 20 kb insert with the upstream region of the T4 g20 homologue from cyanophage strain S-PM2). Tracks contain the following (DNA, enzyme): (1) λ , *Pst*I (marker); (2) pWHW03, *Ssp*I; (3) pWHW03, *Hind*III; (4) pWHW03, *Alu*I; (5) pWHW03, *Cla*I; (6) pWHW03, *Bam*HI/*Eco*RI; (7) pWHW03, *Bam*HI/*Eco*RI; (8) pB063₇, *Pst*I/*Eco*RI; (9) pWHW02, *Bam*HI/*Eco*RI.

(b) Corresponding Southern hybridisation filter probed with a 1 kb *Bam*HI/*Eco*RI fragment, encoding the T4 g20 homologue, from cyanophage strain S-PM2. Hybridisation was at 55°C in 5x SSPE, 0.1% (w/v) SDS overnight, followed by washes at 55°C with 2x SSPE, 0.1% (w/v) SDS for 15 min twice, followed by 0.1x SSPE, 0.1% (w/v) SDS at 55°C for 5 min. pB063₇ is a plasmid, designed by Wilson, containing DNA which has no homology to the probe used for hybridisation, and therefore acts as a negative control (track 8). pWHW02 contains the 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2, and acts as a positive control (track 9). pWHW03 cut with *Bam*HI/*Eco*RI acts as a positive control, since this should give the same size hybridisation band as pWHW02 cut with *Bam*HI/*Eco*RI (track 7).

EcoRI end of the homologue, and was therefore isolated for sequence analysis. pWHW03 was restricted with *ClaI*, and the 1 kb fragment was purified using the GeneClean II kit (2.8.3; 2.8.4), and subsequently sequenced by random subcloning into M13, using the self-ligation and sonication method (2.8.9.1-2.8.9.5). The resulting sequence data reached well upstream of the start of the open reading frame, on both strands (Append. 1a).

To obtain sequence data downstream of the 3' *BamHI* end of the 1 kb fragment it was necessary to isolate a larger fragment likely to contain the whole gene. Hence, the genome of cyanophage strain S-PM2 was restricted with a range of enzymes (2.8.3) and the resulting digests were analysed by Southern hybridisation (2.8.8a) (Fig. 4.2). The enzymes chosen had sites in the pUC19 polylinker and did not cut the 1 kb fragment.

Following restriction digest analysis (Fig. 4.2a) it was revealed that, of the enzymes used, only *AccI* and *XbaI* restricted cyanophage strain S-PM2 DNA. Comparison of the Southern blot (Fig. 4.2b) with the restriction digest (Fig. 4.2a) revealed both a 5.4 kb *AccI* fragment and a 2 kb *XbaI* fragment which cross-hybridised with the probe, suggesting they contained the T4 g20 homologue sequence.

A library of *XbaI* fragments from part of the genome of cyanophage strain S-PM2 was generated in pUC19 (2.8.3-2.8.6). Southern hybridisation analysis (2.8.8b), using the 1 kb *BamHI/EcoRI* insert from pWHW02 as a probe, identified one clone containing two inserts (1 kb and 2 kb) (clone 39; Fig. 4.3). This clone was subsequently found to contain the entire T4 g20 homologue sequence (in the 2 kb insert) and was termed pNJF1. To reduce the sequencing effort pNJF1 was further subcloned to generate pNJF2, which contained only the 2 kb insert with the complete T4 g20 homologue gene sequence.

The 2 kb *XbaI* fragment was purified and ligated to M13mp18 for sequencing (2.8.4, 2.8.9.5). The downstream region of the T4 g20 homologue was subsequently sequenced (2.8.9) using an oligonucleotide primer designed from sequence data

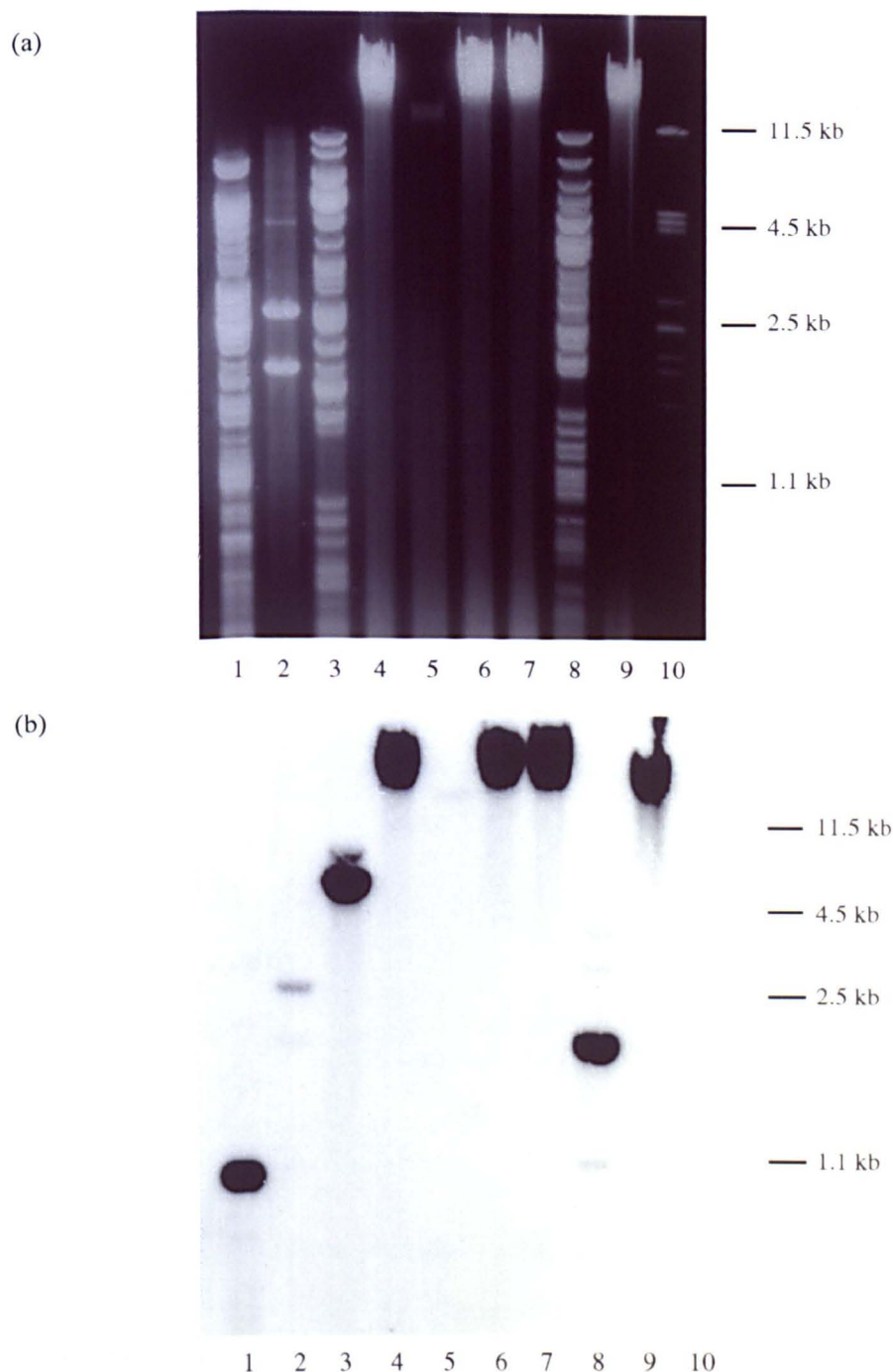
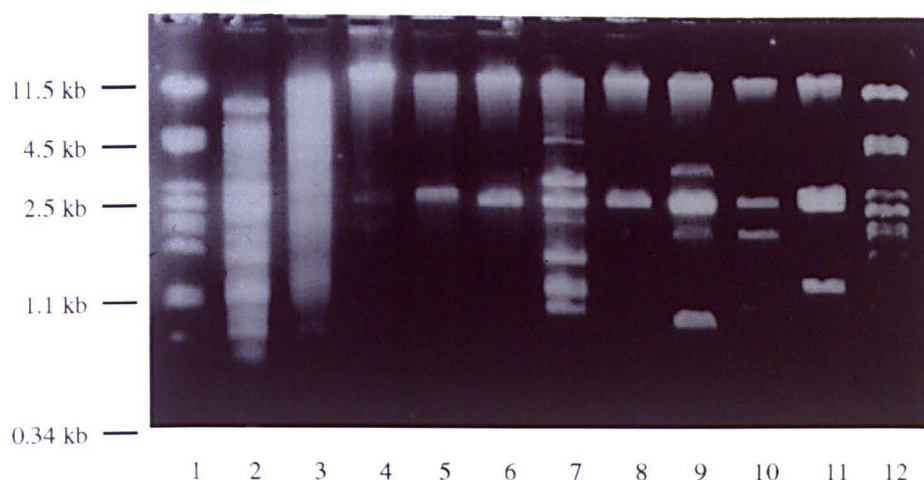


Figure 4.2 Southern analysis of digests of cyanophage strain S-PM2 using the pWHW02 probe.

(a) Restriction endonuclease digestion of the genome of cyanophage strain S-PM2. Tracks contain the following (DNA, enzyme): (1) S-PM2, *Bam*HI/*Eco*RI; (2) pB0637, *Pst*I/*Eco*RI; (3) S-PM2, *Acc*I; (4) S-PM2, *Kpn*I; (5) S-PM2, *Sac*I; (6) S-PM2, *Sal*I; (7) S-PM2, *Sph*I; (8) S-PM2, *Xba*I; (9) S-PM2, *Xma*I; (10) λ , *Pst*I (marker).

(b) Corresponding Southern hybridisation filter probed with a 1 kb *Bam*HI/*Eco*RI fragment, encoding the T4 g20 homologue, from cyanophage strain S-PM2. Hybridisation was at 55°C in 5x SSPE, 0.1% (w/v) SDS overnight, followed by washes at 55°C with 2x SSPE, 0.1% (w/v) SDS for 15 min twice, followed by 0.1x SSPE, 0.1% (w/v) SDS at 55°C for 5 min. Cyanophage strain S-PM2 cut with *Bam*HI/*Eco*RI acts as a positive size control (track 1). pB0637 is a plasmid, designed by Wilson, containing DNA which has no homology to the probe used for hybridisation, and therefore acts as a negative control (track 2).

(a)



(b)

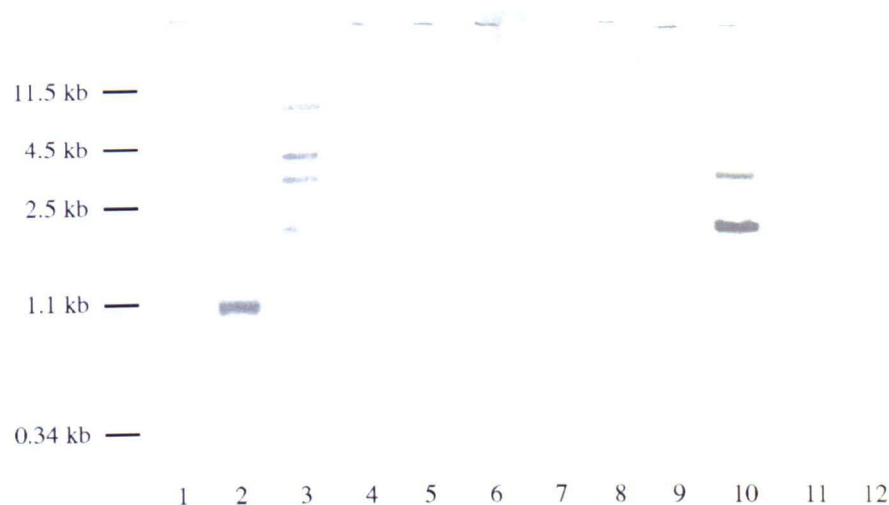


Figure 4.3 Southern analysis of a limited *Xba*I library of cyanophage strain S-PM2 using the pWHW02 probe.

(a) Restriction endonuclease digestion of a limited *Xba*I library of cyanophage strain S-PM2, in pUC19, restricted with *Xba*I. Each clone track contains a single clone from the library. All clones were restricted with *Xba*I. Tracks contain the following: (1) λ , *Pst*I (marker); (2) S-PM2 genome (*Bam*HI/*Eco*RI); (3) S-PM2 genome (*Xba*I); (4) Clone 33; (5) Clone 34; (6) Clone 35; (7) Clone 36; (8) Clone 37; (9) Clone 38; (10) Clone 39; (11) Clone 40; (12) λ , *Pst*I (marker).

(b) Corresponding Southern hybridisation filter probed with a 1 kb *Bam*HI/*Eco*RI fragment, encoding the T4 g20 homologue, from cyanophage strain S-PM2. Hybridisation was at 65°C in 5x SSC, 0.02% (w/v) SDS overnight, followed by washes at room temperature with 2x SSC, 0.1% (w/v) SDS for 5 min twice, followed by 0.1x SSC, 0.1% (w/v) SDS at 65°C for 15 min twice. Cyanophage strain S-PM2 DNA was restricted separately with *Bam*HI/*Eco*RI and *Xba*I and run alongside as positive size controls. The multiple bands larger than 2 kb in the *Xba*I digest of the cyanophage strain S-PM2 genome probably resulted from incomplete digestion of the genome.

upstream of the *Bam*HI site (P1: 5'-ACAAAGCATTTAATCTTG-3') (see Fig. 4.6a). The complementary strand was end-sequenced using the M13 forward (-40) primer (2.8.9). Hence, the entire open reading frame of the T4 g20 homologue in cyanophage strain S-PM2 was sequenced on both strands (Append. 1a). A schematic diagram of the resulting sequence is shown in Figure 4.6a, with an open reading frame of 1692 bp encoding a 564 amino acid peptide sequence (Append. 1a and b).

4.3.2 Isolation of the T4 gene 20 homologue from cyanophage strain S-WHM1

A 3.5 kb *Bam*HI fragment, containing part of the T4 g20 homologue from cyanophage strain S-WHM1, had been cloned into pUC19 to generate pWHW01 (Wilson, 1994). pWHW01 was then restricted with *Bam*HI/*Pst*I in order to release a smaller, 1.4 kb *Bam*HI/*Pst*I fragment (2.8.3), which Wilson had previously shown would cross-hybridise with the 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2 (Wilson, 1994), and therefore still contained part of the T4 g20 homologue. The 1.4 kb *Bam*HI/*Pst*I fragment was then ligated into M13mp18 and M13mp19 and sequenced in the same manner as the 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2 (2.8.9).

Analysis of the sequence data from the 1.4 kb *Bam*HI/*Pst*I fragment revealed that this fragment contained the start of the open reading frame. However, comparison of the sequence with T4 g20 suggested that there could be *ca.* 200 bp of open reading frame downstream of the 3' *Bam*HI end. Hence, an additional DNA fragment had to be cloned from cyanophage strain S-WHM1 to complete the sequence of the gene. For a schematic diagram of the T4 g20 homologue region, see Figure 4.6b.

Previous research (Wilson, 1994) showed that digestion of DNA from cyanophage strain S-WHM1 with *Eco*RV resulted in a single 3 kb fragment which cross-hybridised with the 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2.

Analysis of the sequence data already obtained for the S-WHM1 1.4 kb *Bam*HI/*Pst*I fragment showed an *Eco*RV site 540 bp downstream of the start codon. It is surprising that only a single fragment cross-hybridised; however, it may be that the two *Eco*RV fragments, containing either half of the T4 g20 homologue, are both the same size.

To obtain the *Eco*RV fragment which contained the 3' end of the homologue, a library of *ca.* 3 kb *Eco*RV fragments from cyanophage strain S-WHM1 was generated in pUC19 (2.8.5-2.8.6). Southern hybridisation analysis (2.8.8b), using a 165 bp PCR product as a probe, identified a 3 kb insert (clone 12; Fig. 4.4) which was subsequently found to contain the 3' end of the homologue gene sequence. This clone was termed pNJF4. The PCR product, described later in this chapter, was amplified from the region downstream of the *Eco*RV site (2.8.10.1) (Fig. 4.6b).

The 3 kb *Eco*RV insert was purified from pNJF4 (2.8.4) and ligated to M13mp18 and M13mp19 for sequencing (2.8.9). The 3' region of the T4 g20 homologue was subsequently sequenced downstream of the *Bam*HI site, to beyond the stop codon, using an oligonucleotide primer (P2: 5'-ACCATGTTTCAGTTTGATT-3') designed from sequence 85 bp upstream of the *Bam*HI site (see Fig. 4.6b). From this sequence data a second primer (P3: 5'-TTTTCGTCACCTATACACT-3') was designed, from the region downstream of the stop codon (Fig. 4.6b), in order to sequence the complementary strand. Hence, the entire open reading frame of the T4 g20 homologue in cyanophage strain S-WHM1 was sequenced in both strands (Append. 1c). A schematic diagram of the resulting sequence is shown in Figure 4.6b, with an open reading frame of 1602 bp encoding a 534 amino acid peptide sequence (Append. 1c and d).

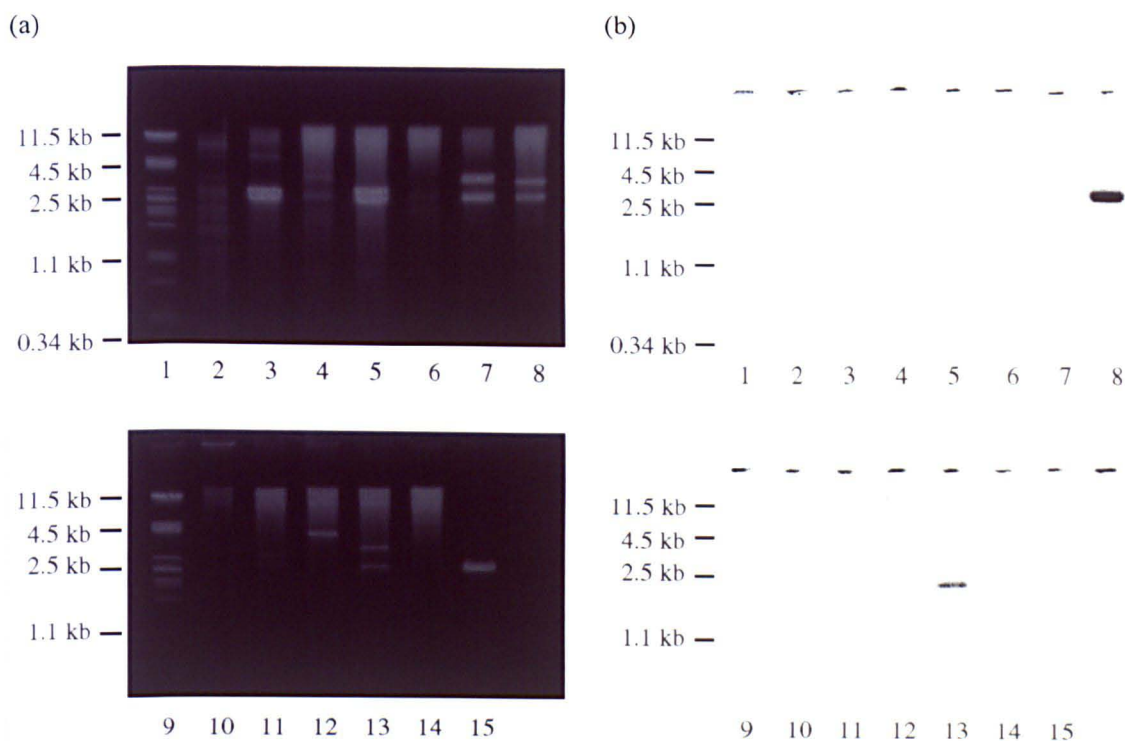


Figure 4.4 Southern analysis of a limited *EcoRV* library of cyanophage strain S-WHM1 using a PCR product probe.

(a) Restriction endonuclease digestion of a limited *EcoRV* library of cyanophage strain S-WHM1, in pUC19, restricted with *SacI* and *HindIII*. Each clone track contains a single clone from the library. Tracks contain the following: (1) λ , *PstI* (marker); (2) S-WHM1 genome (*EcoRV*); (3) Clone 1; (4) Clone 2; (5) Clone 3; (6) Clone 5; (7) Clone 11; (8) Clone 12; (9) λ , *PstI* (marker); (10) Clone 4; (11) Clone 6; (12) Clone 7; (13) Clone 8; (14) Clone 10; (15) pUC19 (*EcoRV*).

(b) Corresponding Southern hybridisation filter probed with a 165 bp PCR product amplified from part of a T4 g20 homologue, from cyanophage strain S-WHM1. Hybridisation was at 65°C in 5x SSC, 0.02% (w/v) SDS for 6 h, followed by a wash at room temperature with 2x SSC, 0.1% (w/v) SDS for 10 min, and then with 0.1x SSC, 0.1% (w/v) SDS at 65°C for 15 min. Cyanophage strain S-WHM1 and pUC19 DNA were restricted separately with *EcoRV* and run alongside as positive size controls.

4.3.3 Isolation of the T4 gene 20 homologue from cyanophage strain S-BnM1

A 1.5 kb *EcoRI/PstI* fragment, containing part of the T4 g20 homologue from cyanophage strain S-BnM1, had been cloned into pUC19 to generate pWHW04 (Wilson, 1994). This 1.5 kb insert was ligated into M13mp18 and M13mp19 and sequenced in the same manner as the 1 kb *BamHI/EcoRI* fragment from cyanophage

strain S-PM2 (2.8.9). Analysis of the resulting sequence data revealed that this fragment contained the start of the open reading frame; however, comparison of the sequence with T4 g20 suggested that there could be *ca.* 200 bp downstream of the 3' *Pst*I end. Hence, an additional DNA fragment had to be cloned from cyanophage strain S-BnM1 to complete the sequence of the gene. For a schematic diagram of the T4 g20 homologue region, see Figure 4.6c.

Previous research (Wilson, 1994) showed that digestion of DNA from cyanophage strain S-BnM1 with *Eco*RI resulted in a single 5.1 kb fragment which cross-hybridised with the 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2. Hence, a library of *Eco*RI fragments was generated in pUC19 (2.8.5-2.8.6) and, following further Southern hybridisation analysis (2.8.8b), using the 165 bp PCR product from cyanophage strain S-WHM1 as a probe (4.3.2), a clone containing a 5.1 kb insert was identified (clone 47; Fig. 4.5). This clone was subsequently found to contain the entire T4 g20 homologue sequence and was termed pNJF5.

The 5.1 kb *Eco*RI insert was purified from pNJF5 (2.8.4) and ligated to M13mp18 and M13mp19 for sequencing (2.8.9). The 3' region of the T4 g20 homologue was subsequently sequenced downstream of the *Pst*I site, to beyond the stop codon, using an oligonucleotide primer (P4: 5'-GAAGATATGAAGGAGTAC-3') designed from a region 90 bp upstream of the *Pst*I site (see Fig. 4.6c). From this sequence data a second primer was designed (P5: 5'-CAACGCTGATGCATGTTT-3'), from the region downstream of the stop codon (Fig. 4.6c), in order to sequence the complementary strand. Hence, the entire open reading frame of the T4 g20 homologue in cyanophage strain S-BnM1 was sequenced on both strands (Append. 1e). A schematic diagram of the resulting sequence is shown in Figure 4.6c, with an open reading frame of 1632 bp encoding a 544 amino acid peptide sequence (Append. 1e and f).

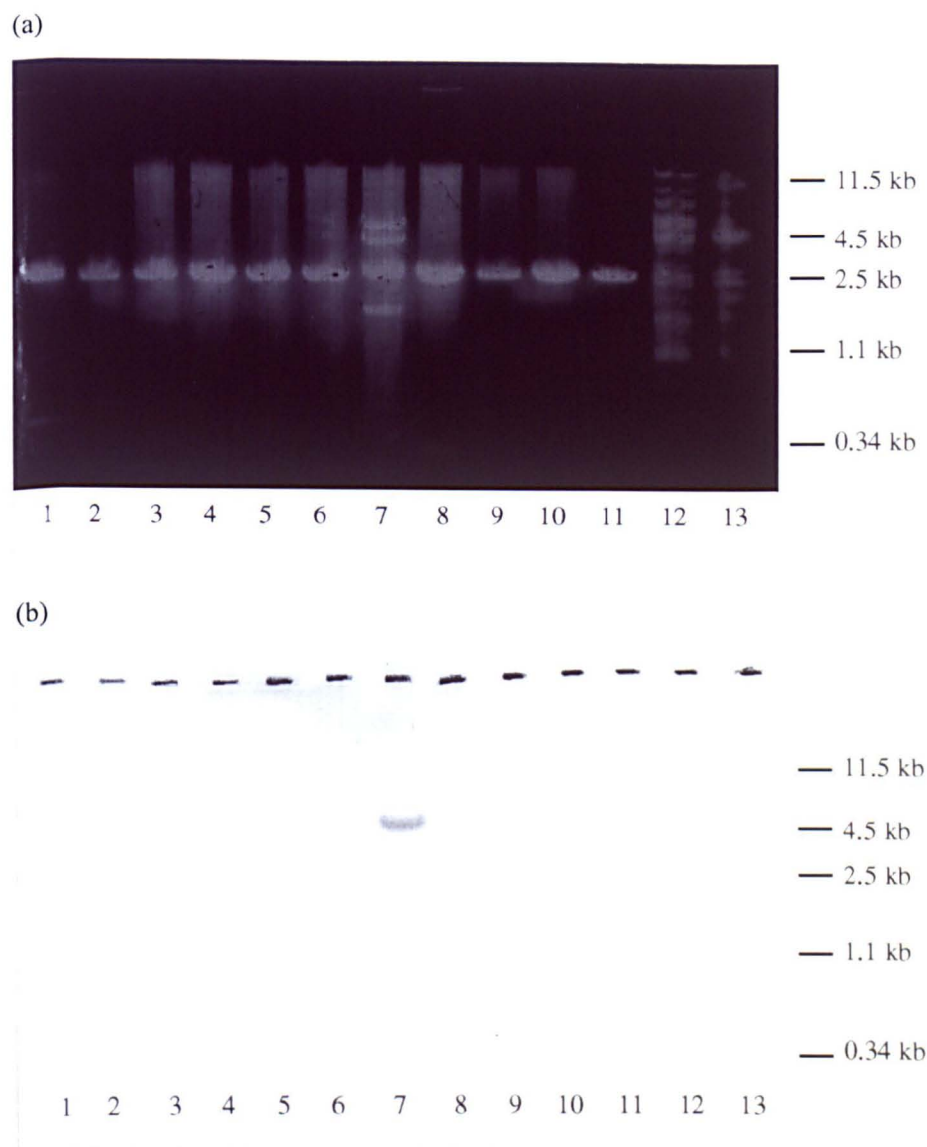


Figure 4.5 Southern analysis of a limited *Eco*RI library of cyanophage strain S-BnM1 using a PCR product probe.

(a) Restriction endonuclease digestion of a limited *Eco*RI library of the cyanophage strain S-BnM1, in pUC19. Each clone track contains a single clone from the library. Tracks contain the following: (1) Clone 41; (2) Clone 42; (3) Clone 43; (4) Clone 44; (5) Clone 45; (6) Clone 46; (7) Clone 47; (8) Clone 48; (9) Clone 49; (10) Clone 50; (11) pUC19 (*Eco*RI); (12) S-BnM1 genome (*Eco*RI); (13) λ , *Pst*II (marker).

(b) Corresponding Southern hybridisation filter probed with a 165 bp PCR product amplified from part of a T4 g20 homologue, from cyanophage strain S-WHM1. Hybridisation was at 55°C in 5x SSC, 0.02% (w/v) SDS overnight, followed by a washes with 2x SSC, 0.1% (w/v) SDS at room temperature for 10 min, then at 55°C for 15 min. Cyanophage strain S-BnM1 DNA and pUC19 DNA were restricted separately with *Eco*RI and run alongside as positive size controls.

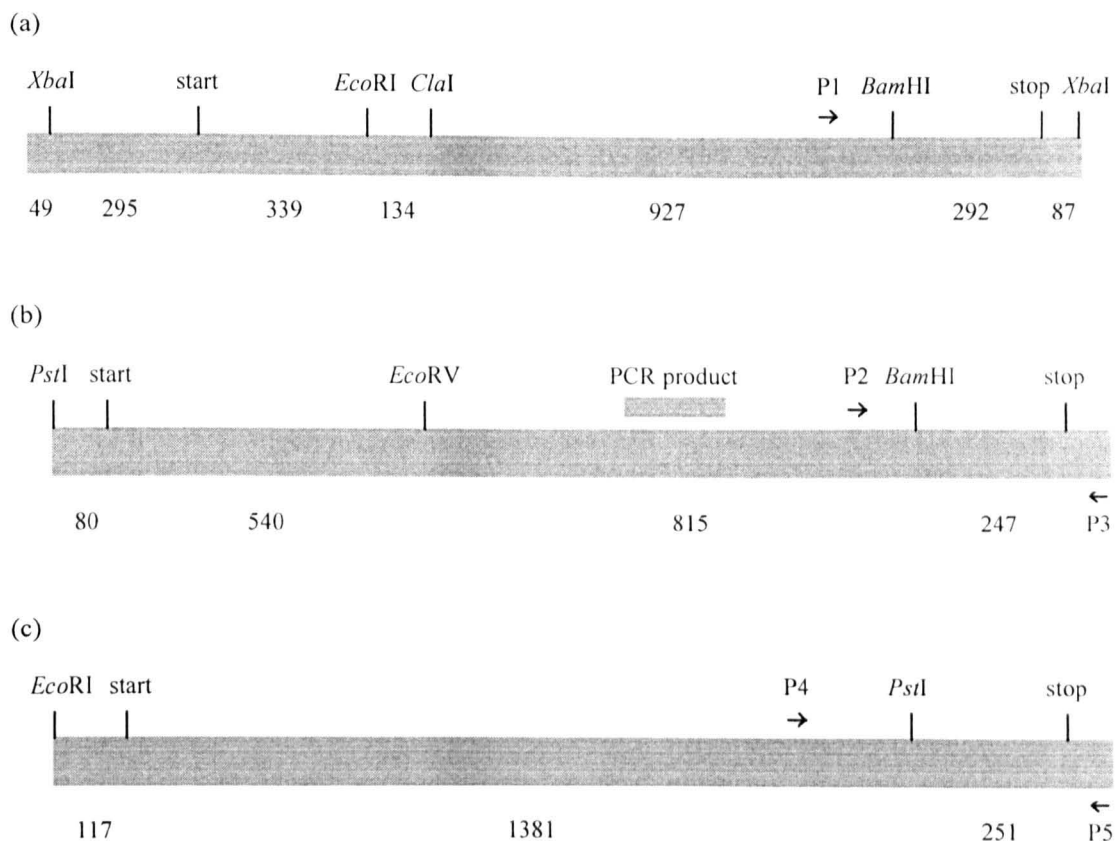


Figure 4.6 Partial restriction maps of DNA sequence data obtained for T4 g20 homologues in three cyanophages. The start and stop codons of the open reading frames (ORFs) are marked, as are relevant restriction sites. Numbers below the fragments represent distances in bp. (a) Cyanophage strain S-PM2; 1692 bp ORF. P1 is a primer used for sequencing downstream past the *Bam*HI site. (b) Cyanophage strain S-WHM1; 1602 bp ORF. P2 and P3 are primers used for sequencing the 3' terminus of the homologue, downstream past the *Bam*HI site. The PCR product was used as a hybridisation probe to detect an *Eco*RV fragment containing the 3' end of the homologue. (c) Cyanophage strain S-BnM1; 1632 bp ORF. P4 and P5 are primers used for sequencing the 3' terminus of the homologue, downstream past the *Pst*I site.

4.4 Sequence Data and Analysis

Having cloned DNA fragments spanning the entire T4 g20 homologues in all three cyanophages, complete double-stranded sequence data were obtained. The DNA and amino acid sequences are displayed in Appendix 1. Accession numbers are: AFO16384 (cyanophage strain S-PM2); AFO16385 (cyanophage strain S-WHM1); and AFO16386 (cyanophage strain S-BnM1).

Analysis of the sequence data was made by comparing the three cyanophage sequences with each other, and also with the T4 sequence (accession number P13334). The software package GCG was used to perform alignments of the sequences and to calculate the degrees of similarity (2.8.9.11). "Gap" alignments of all the sequences of cyanophages with each other and with T4 revealed the degree of similarity at both the DNA and amino acid levels, as shown in Table 4.1.

Table 4.1 Similarities between T4 g20 homologues in three cyanophage strains (S-PM2, S-WHM1 and S-BnM1) and T4. Similarities, at both the DNA and amino acid levels, were revealed by "Gap" alignments, using the software package GCG. Similarity was calculated only over the region of the open reading frame.

Viruses	DNA	Amino acid	
	% identity	% identity	% similarity
S-PM2 + S-WHM1	60.1	59.0	75.4
S-PM2 + S-BnM1	62.7	61.5	78.3
S-WHM1 + S-BnM1	63.5	61.5	77.6
S-PM2 + T4	50.9	35.7	60.4
S-WHM1 + T4	49.5	38.6	62.8
S-BnM1 + T4	50.6	38.4	61.8

GCG was also used for making a "Pileup" alignment, to align all the sequences from the three cyanophages and T4. Figure 4.7 shows this "Pileup" for both DNA and amino acid sequences, revealing conserved and variable regions.

A recent study (Monod *et al.*, 1997) revealed that the coliphage RB49, termed a pseudo T-even phage, also possessed a T4 g20 homologue (accession number Z78091). The sequence data for this homologue is very limited; however, a "Pileup" alignment was made of this sequence together with the three cyanophage and T4 sequences (Fig. 4.8). "Gap" alignments revealed the percentage similarity between some of these viruses over this region (Table 4.2).

(a)

S-PM2 : GCAATCAACACTCACAACCAACAACGCTCCTCTATTTCACCTCCAAAGTCTAGACTCTGTTATGCCAGAATCTCAGTTAGCACTGAGAGATG : 90
S-WHM1 : : -
S-BnM1 : : -
T4 : : -

S-PM2 : AAGTGAGTCGGGTGAAGTTCITAGAGAGTATGTTCTCTACTACGGATTCCCAACTAATGTTCTCAAATTGATGTTGATATGACAGCAAC : 180
S-WHM1 : : -
S-BnM1 : : -
T4 : : -

S-PM2 : GATCAGATCGAGGAATTCACAGTTGAGTTCCAACTACTCATCTGGAGGTTGAATCTGTACTACACAAAATGGAGTATCTGGAGTCAAG : 270
S-WHM1 : : 11
S-BnM1 : GAATCCAAAGTTCAGTGGTGGCAAGCAGAGCAGGCAITGAAGGTGGTAA : 51
T4 : GCITAAAGCAGAGAAGTCCATATATAATATA : 31

S-PM2 : TCGGGAATCGGTGATCAGCGCTGTAGAGACTGTAAATAGTCTTAAGGTAGGTCTACTATATTACTATGAGTCAACTCTTGGT : 360
S-WHM1 : GTATTGGACTCCGATTATAGCAACCCACTGACCTGTAAATAGACAGGACCAA...TAACITAGAAATATAATGTCTCAGCTCTCGGAT : 99
S-BnM1 : ACCATCGGTGAGCGAAGTTCGTACCTGACTAAATGGATATGATTCGATT...CACATATTTTGGAGATCCCGAATTCCTGGAT : 139
T4 : ACTATAATTCCTATTGGAGAAATCAATGAATTTATGTATTAGTTTGGTTGCTCCATGGGCTAAATGGACCAACGAATTTAAAG : 121

S-PM2 : TTTTATAAA...TGAAGAGAGGACAGAAAGGTCAATCCCTTCTCTCCAA...GATGAACATCCGTCAGTACTG : 435
S-WHM1 : TTTCTCTTGAAGAGG...GAAGAGGTCCCAAGGACCTCTTT...TTTGAAGAGATAGCATGGATGTTCCGACCTTATG : 180
S-BnM1 : ATTCTATCAACGAGGAGAACAGAAAGGTGGCAAAAAATCCATCTCTCTTCTCTCCAA...GAAGACGATGGCAAGTATCTATAG : 229
T4 : ACCAGAGAAAGAGATCTTGTTCATTACACCCCAAGCTTGTATGATGGAAACAGAGATT...GAAGTAAGCTCGAATCAAGCTGCTT : 211

S-PM2 : TTGGTGGCGGATATTTGGTACTTATGTTATACATCTCTGTGACAAATTCAGAAACGAAATCTAACCTCATCTGTAGATATCTGTT : 525
S-WHM1 : TAGCGGGCGGATACTACGGATACTCTGTCGATTTCTGACGGAC...GGTACGTATGATTTATGACTAAATCCCGTTATAGGAGA : 264
S-BnM1 : CTGCTGGTGGCACTATGGTACTTTTTCGATCTAGAAGGTGG...TGGGAAAACGAACAGCACTTTTGAAGCTTATCTGTAGA : 313
T4 : CTCTTATAATGCTGCATCTCCAAACAAATTTTGGTTCTATGAACAGGAATGAAATCTACTCTGTAAGCTTATGATATATCTTAATC : 301

S-PM2 : TGTCTCTCCACCGCAAGTGTATAGTTCATAGACAAATCGGAATAGTTTGTTTAAAGATGGCGCGAATAACCCCTAGAGATG : 615
S-WHM1 : TGGTTCTACACCCGAGTGTATAGTGTGTATATATTCACCGAACTATTTGGGAACTTTGATGATCTTACCTTGTGATGG : 354
S-BnM1 : TGGCACTCTCCCTTGAAGTGTATGGTGCATCGAAGATATATCAATGAAAGCCGCTAAGGTCTGTGTATAGTCCCTACAGATC : 403
T4 : CATGAATACTATAGTATATAGTGTATTCAGAAATCGTTCCAGTGTATCTGTATAGAGATGATCTCAAGTCTAGCGTAA : 391

S-PM2 : ATCTCAAACTTAGAATTCGATCTGGTCAAGAAATAATTCGCGATGAATTCATAGAAATCTTCGTATGAGAACTTTATGTCT : 705
S-WHM1 : AACTTTCAACCTGAGGGGTGGGATATAAATACTTATGAGAGAGGAGTTTCAGCAAGTCTCTCGCTCCCTGATTTGAAAAATC : 444
S-BnM1 : AACTTTCAAACTTAGACCGCAAGTAAATAAGTAAGTAAGTCTTATCAGAGAGAGTTTGAATATCAAGAACTCTCAACTTGTATAAA : 493
T4 : ATTGGATATCTTAATTTAGCCCAAAATAAATAATATAGTTTATGATGAATTAGTGTGATTAATTAATATCTATCCCTTACAA : 481

S-PM2 : AATCACATGAAATTAACAATAACCTGATTTGATGGCTCACAATACCAACAGAAATTAATCTTAGTAATCAAGAAAGATATCT : 795
S-WHM1 : GTTCGTATGAAATCTCCCTAGAGGTATTCGATGGAAGACTCTTACCATTAAGCAATCTATCCCAAAATTCCTGGTGTCTG : 534
S-BnM1 : AATCACATGAAATCTCCCGGTGGTACCTGATGGAAGACTTTATATATATAAAGATTAAGTTTGTATGCTCTTCAAGCAAAATA : 583
T4 : AAGTTCATATCATTTATACCTTGGTATGTTATATCAAGAAATTTCTTTTATATAAATCTCTATCCAAACCTGCAAAAGAACATA : 571

S-PM2 : TAGAATTAATATACATCGATTCTTAAAGATAGAAAGTTAGGCAAAATTAAGAGCTAGATCCCAATAGAAAGGAATAGAAAGG : 885
S-WHM1 : CAGAACTTCGCTATATCGATCTTGGCAATCTGTAAGT...AACTGAGTCCGAACGAAGCGCCCTGAGCAACTCTGTTGGC : 615
S-BnM1 : CAGAGATCATATATGCTATCAATAAATAATAGACTTT...AA...AAGAGTACAGAAAAATCGAGATCAGACGAGAAGT : 661
T4 : AAGAAATCTAGATAGACCTTGGCACTCAGTATCTCTGAAATTTAACAGAA... : 630

S-PM2 : GAACCTGCTCTCAATATGATACGGACCTTCATCAATATATATTTTCAATCCAAAGGATTTGCAGTAAATCCCAATGTTACTG : 975
S-WHM1 : TTGATATCAATATCTACTACACAGAAAGCAGAGATTTCTTTTCAACCCCAAGGTTTGAAG... : 685
S-BnM1 : ATCTGAATAACAGTATGATACGAAATGCTACTTGAATATACATCAATCCAAAGGCTCGAAGCAGAAATCCCAATGAAGTCTCT : 751
T4 :CTGAGCTGGCAAAATATAGTTAAGGTACAAAGATATTTTATATAGATCTGCCATGAGTATATGATG : 709

S-PM2 : CTTCGATGGATTTGGTCAACCAAGAGAGTATTAATAATGCTGCAATGTTACGGCAATCACTCTCTTTGAGTGAATCAAAACAA : 1065
S-WHM1 :CTTACTAATCAGGTATGAAAAATACCTTATTTGTTTCTGATGTTACTTAAATTAAGCACTGAATAAA : 762
S-BnM1 : CTGC...ACCAACTGGTGGTGTATCAAAATGCAAAAGAGAAACACTTTTGTACAGTCTGCTCTCTTTGAGCAACAAAG : 832
T4 : AATGTTGAATGTATGAGCTGGCACAAAAATAAATAATCTTAAACCTGCGCTTTATGCCATTTGATATAGTCAATGTTTGGT : 799

S-PM2 : AATGACATTAAGCTTTCTATAAGCAATCAAGTTCTCAATTAACTAAGAAATGATCTAGAGTACTCTTTATCTATCTTTATCT : 1155
S-WHM1 : AATGACTCTTAGTCTCCATACATAAGCGATTAAGGAGCTCAACCAACAAAGAAATGATCAAGACTCTTGGTTATCTACGTTTGA : 852
S-BnM1 : GTTGTGTTCTTATACCTCCATATAAGCAATCAAGACTCAATCAACTCTCAATGATTCAGGACTCTTGGTCTATCTATCTTTAT : 922
T4 : AATATCAACCGCTATTGCTATCTGTTGTTAACTTGCTAACCAATTAATTAATTAAGATGTGTAAGTCAATTAATGCAATACT : 889



Figure 4.7 "Pileup" alignment of the sequences of T4 g20 homologues in three cyanophage strains (S-PM2, S-WHM1 and S-BnM1) and T4. Alignments are made at both the DNA and amino acid levels, using the software package GCG. Conserved bases and residues are highlighted to four levels. (a) "Pileup" alignment of DNA sequences. (b) "Pileup" alignment of amino acid sequences (continued overleaf).

(b)

S-PM2	:MSQLPGFLIN...EKEGQKQSPVPND.EA.....SVSTVAGGYFGTYVDTSGG	:	46
S-WHM1	:MSQLPGFSLERA...KKVPKGFSPVQKDSMDG.....SQPIVGGGYGYGSVDPDGT	:	48
S-BnM1	:MPLEPGYSIKRAEQERGKKSTSPVPNEDDG.....QVSTAAAGGHYGYFVDLEGG	:	51
T4	:	MKFNVLSLFAPWAKMDEENFKDQEKEDLVSITAKLDDGAREFEVSNEAASPYNAAFQTFGS	:	64
S-PM2	:	..QNSRNEYELIRRYRDMSLHPEVDSATIDEIVNEFVUNDGDDKFEVDLQNLKIGSGVKKKIRD	:	108
S-WHM1	:	...VRNDYELISRYREMLQPECDASVDDIVNETICGNFDDVPVELELSNLKASDKIKKLMRE	:	108
S-BnM1	:	...GKNEQELIRRYREMLHPEVDGATEDIINEAVVSDLYDSPVOIELSNLDASNKVKKLIRE	:	111
T4	:	YEPGKMTTRELIDTYRNLMNNEYVDNAVSEIVSDAIVYEDDTEVVALNLDKSKFSFKIKNIMLD	:	128
S-PM2	:	EENRILRMNPNVNAHEIIRNYYVDGRSHYHKVIDLDNPKKGILELRYIDSLKIRKVRQKLKDV	:	172
S-WHM1	:	EFDEVLRLLDENRAYEIFRRWYVDGRLFYHKVIDQNPFGGLAELRYIDPRKIRKV...TEY	:	168
S-BnM1	:	EFEYTKKLLNFDKKAHEIFRRWYVDGRLYYHKMIDFDDHSGKITEIRYVDPQKIRLV...KEV	:	171
T4	:	ESDVLNHLSPQKQGSDFRRWYVDSRIFPHKIIDPKRPEKIGIKELRLDPRQVQYVREITET	:	192
S-PM2	:	DPNRKEIEKGTALQ.YDYGDFIEYYIYNPKGFAGNIPMVTGSMDSNQEGIKIASDATAQSTSG	:	235
S-WHM1	:	EOKRPEQLRGVDINTQLTQKAAEYFLYNPKGLKN.....STNOGKMITTDSVCYCHSG	:	221
S-BnM1	:	..QKNADQTRTDLNNKYDGNVLEYYIYNPKGLKN...PMKSATAPTGGGKIAKDAITVQSG	:	231
T4	:	EA.....GTKIVKGYKEYFIYDT...AHESYACDGRMYEAGTKIKPKAAVVAHSG	:	241
S-PM2	:	LMDLNKKMTLSLHKAIKSLNQLRMIEDSLVIYRLSRAPERRIFYIDVGNLPKVKAEQYLKDV	:	299
S-WHM1	:	LQDLNKNMTLSLHKAIKAVNQLRMIEDSLVIYRLSRAPERRIFYIDVGNLPKVKAEQYLREVM	:	285
S-BnM1	:	LLDANKGMVLSYLHKAIKALNQLRMIEDSLVIYRLSRAPERRIFYIDVGNLPKVKAEQYLREVM	:	295
T4	:	LVDCCGNIITGYLHRAVKPANQLKLEDAVVIYRITRABDRRVYVDTGNMPARKAAEHMQHVM	:	305
S-PM2	:	SRYRNKLVDGQTGEIRDDKKHMSMLEDFWLPRREGGRGTEITLPGGQNLGELKDVEYFKKKL	:	363
S-WHM1	:	SRYRNKMVYDANTGEIKDDKKFMSMLEDFWLPRREGGRGTEISTLPGGQNLGELEDVKYFKKKL	:	349
S-BnM1	:	GRYRNKLVDYDANTGEIKDDKKHMSMLEDFWLPRREGGRGTEISTLPGGQNLGELTDVEYFKKKL	:	359
T4	:	NTMKNRVYDASTGKIKNQQHNSMTEDYWLQRRDGKAVTEVDTLPGADNTGNMEDIRWFRQAL	:	369
S-PM2	:	YNSINLEPSRLT.DDNKAFNLGKSTEILRDELKFTKFIGRLRKRFADLFHILKTOLILKGIT	:	426
S-WHM1	:	YKALNVPGSRL..ETETTFNIGRAAEITRDEVKFKQFIARLRKRFSELFMDLLKTOLILKGVLT	:	411
S-BnM1	:	YRALNVPISRM..EAEGGFNLGRSSEILRDEIKFTKFGVRLRKRFSEIFIDMLKTOLILKGITS	:	421
T4	:	YMALRVPLSRIPQDQGGVVMFDSGTSTIRDELTEAKFIRELQHKFEVFLDFLKTNLLKGIT	:	433
S-PM2	:	PEDWDDMEEHIOYDFLFDNHFNELKEQEQMLQRVNLATQMPFVGKYFSTEYIRKKILMOTENE	:	490
S-WHM1	:	LEEWEEMKNHVQFDYVADNYFTTELKEIEIRNERMNQVNVMDPYVGKYFSVEYMRQVLKQTEQE	:	475
S-BnM1	:	VEEWEDMKKEYIQDFIYDNHFSELKESELLQERINLASAADPYVGKYFSVEYVRSKILHOTDE	:	485
T4	:	EDWNEINNTKIEFHRDSYFAELKEAEILERRINMLTMAEPPFGKYISHRTAMKDILOMTDEE	:	497
S-PM2	:	FKEIDKQMKSDIESGLAIDFIVNMLDDMEKQNAFAPELQAAQDDLAAEREIKKLNSAPKPPP	:	554
S-WHM1	:	IEEIDKQIESEMESGI.TADPAAEMDPAMAAGNEG.....G...GAPAAEVAPN..ESAVEPAD	:	528
S-BnM1	:	MDMDDDQIEKEKDAGI..TPFSEEM.MAMQGGEG.....GALGDVPMDFEAD..GSATERPE	:	539
T4	:	IEQEAQIEEESKEARFQDFDQEQEDF.....	:	524
S-PM2	:	SQSKSQSNK*	:	564
S-WHM1	:	ARRGETI*....	:	534
S-BnM1	:	G..QGLI*....	:	544
T4	:	:	-

Figure 4.7 (continued) "Pileup" alignment of the sequences of T4 g20 homologues in three cyanophage strains (S-PM2, S-WHM1 and S-BnM1) and T4. Alignments are made at both the DNA and amino acid levels, using the software package GCG. Conserved bases and residues are highlighted to four levels. (a) "Pileup" alignment of DNA sequences. (b) "Pileup" alignment of amino acid sequences.

(a)

```

S-PM2 : TTTTATATAA ..... TGAAGAAGAGGACGAGAAAGGTCATCCCGTTCCTCCAAAT... GATGAACATCCGTCAGTACTG : 435
S-WHM1 : TTTCACTTGAAAGAGC..... GAAGAAGGTCCCAAGGGACCTTCCTT... TGTTCAGAAAGATAGCATGGATGTTCCGCAACCTATTCG : 180
S-BnM1 : ATTCAATCAAGCGAGCAGAACAGGAAAGAGGTGGCAAAATTCATCTCTCCGTTCCTCCAAATGAAGACGATGACAACTTCCATAG : 229
T4 : ACCAAGAAAAGAAGATCTTGTTCATTACAGCCCCAAAGGCTTGATGGACCAAGAGAAATTGAAGTAAGCTCGAATGAAGCTGCTT : 211
RB49 : ..... AAAACAATGATGGGCTAGGGAAGTAGAAATTAACATAATTCCTGCTA : 51

S-PM2 : TTGCTGGCGGATATTTTGGTACTTATGTTGATACATTTGGTGGACAAAAATTCAGAAACGAATACCAACTCATTCGTAGATATCGTGATA : 525
S-WHM1 : TAGGTGGCGGATACTACGGATACCTCTCTCGATTTTCAGCGGGAC..... GGTACGTAATGATATTAACAAATTTCCCGTTATAGGGAGA : 264
S-BnM1 : CTGCTGGTGGGCACTATGGGTACTTTCTCGATCTAGAAAGGTG..... TGGGAAAACGAACGGAACCTTTGAGACGTTATCTGTGAGA : 313
T4 : CTCTTATAATGCTGCATTCCAAACAATTTTGGTTTATATGAACAGGAATGAAAACCTACTCGTTAGCTTATGATACATATCGTAAATC : 301
RB49 : GCTGTTGAACCTCTTACCCGCAATTTTCAGCA..... CACAGAAAAACGCAACAACAGCAATTAGTTAACACATATCGCGCTT : 138

S-PM2 : TGTCTCTCACCCAGAAAGTGATAGTGCCATAGACGAAATCGTGAATGATTTCGTTAAATGATGGCGACGATAAACCCGTAGAAGTTC : 615
S-WHM1 : TGGTCTTACAACCCGAGTGTGATAGTGTGTTGATGATATTTCACGAAATTAATTGTGGAACTTTTATGATGTACCTGTTCAGTTCG : 354
S-BnM1 : TGGCACTTCACCCCTGAGTGGATGGTGGCATCGAAGATATTATCAATGAAGCCGCTTAAGTGCATCTGATGATAGTCCCGTACAGATCG : 403
T4 : TCAATGAATAACTATGAAGTAGATAAATGCAATTCAGAAATCTTTCAGATGCTATCTGTTATGAAATGATACATGAAGTCTGAGCGTTAA : 391
RB49 : TGATGAATAACCATGAAGTCCAAACGCTGTACAAAATATCTTAACTGAGATGGGATTGTATTGAAGAACCTCATGAAGTTTTCATTAA : 228

S-PM2 : ATCTTCAAAACTTAGAAATTCGATCTGGTCTAAAGAAAAAAATTCGGATGAATTCATAGAAATCTTCGTATGATGAACCTTAAATGTCA : 705
S-WHM1 : AACTTTCCCAACCTGAAGGCGTCGGATAAAATCAAAAACCTTATGAGAGAGGAGTTGACGAAGCTCTTCGCTCCTCGATTTTGAATAATC : 444
S-BnM1 : AACTTTCAAACTTAGCGCAAGTAATAAAGTAAAGAAACCTTATCAGAGAGAGTTTGAAGTATATCAAGAAACTCCCTCAACTTTGATAAAA : 493
T4 : ATTGGATTAATCTTAATTTAGCCCAAAATTAATAATATATGATTTAGATGAATTTAGTATCTATTAATCATCTATCTCTTCAACAAA : 481
RB49 : ATCTTGAGCGGACTTGCTTTAGTCGAATCTCTAAAGAGCGCATTCATCGAAGAAATTAA : 287

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(b)

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S-PM2 : .....MSQLFGFLIN....EKEGQKGQSPVPND.EASVST.....VAGGYFGTYVDT..S : 44
S-WHM1 : .....MSQLFGFSLERA...KKVPKGPSFVQKDSMDGSOPI.....VGGGYGYGVDF..D : 46
S-BnM1 : .....MPELFGYSIKRAEQERGGKKSISPVPPNEDDQVSI.....AAGGHYGYFVDL..E : 49
T4 : MKFNVLSLFAFPAKMDERNFKDQEKEDLVITAPKLDDGAREFEVSSNEAASPYNAAFQTIFGS : 64
RB49 : .....NNDGATEVEINDNSPASSWNSLTQQFY.S : 28

S-PM2 : GGQNSRNEYELIRRYRDMSLHPEVDSADDEIVNEFVUNDCDDKPEVVDLQNL EIGSGVKKKTRD : 108
S-WHM1 : GT..VRNDYELISRYREMVLQPECD SAVD DIVNETICGNFDDVPVELELSNLKASDKIKKLMRE : 108
S-BnM1 : GG..GKNEQELLRRYREMA LHPEVDGAIEDIINEAVVSDLYDSPVQIELSNLDASNKKVKKLIRE : 111
T4 : YEPGMKTTRELIDTYRNL MNNEVDNAVSEIVSDAIVYEDDTEVVALNLDKSKFSPKIKNIMLD : 128
RB49 : TDQKISTTKQLVNTYRGLMNNHEVENAVQNI VND AIVFEEGHEVVS LNL EATGFS ESVKERLHE : 92

S-PM2 : EFNRI LRMMNFNVNAHEIIRN WYVDGRSHYHKVIDLDNPKKGILELRYIDSLKIRKVRQKLKDV : 172
S-WHM1 : EFDEVLRLLDFENRAYEIFRRWYVDGRFLYHKVIDPQNPRGGLAELRYIDPRKIRKV...TEY : 168
S-BnM1 : EFEYIKKLLNFDKKAHEIFRRWYVDGRFLYHKMIDFDDPSKGITEIRYVDFQKIRLV...KEV : 171
T4 : EFSVDLNLHLSFQKGSDFRRWYVDGRIFFKIIPKRPKEGIKELRLDPRQVQYVREITET : 192
RB49 : EFK..... : 95

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Figure 4.8 "Pileup" alignment of partial sequence data of T4 g20 homologues in three cyanophages, T4 and RB49. S-PM2, S-WHM1 and S-BnM1 are cyanophage strains; RB49 is a pseudo T-even coliphage (Monod *et al.*, 1997). Alignments are made at both the DNA and amino acid levels, using the software package GCG. Conserved bases and residues are highlighted to four levels. (a) "Pileup" alignment of DNA sequences. (b) "Pileup" alignment of amino acid sequences.

Table 4.2 Similarities between T4 g20 homologues in two cyanophage strains (S-PM2 and S-WHM1), T4 and RB49. Similarities, at both the DNA and amino acid levels, were revealed by "Gap" alignments, using the software package GCG. Similarity was calculated only over the region for which RB49 sequence data were available. RB49 is a pseudo T-even coliphage (Monod *et al.*, 1997).

Viruses	DNA	Amino acid	
	% identity	% identity	% similarity
S-WHM1 + S-PM2	52.1	49.0	68.8
S-WHM1 + T4	41.0	28.1	50.0
S-WHM1 + RB49	40.8	25.3	50.5
T4 + RB49	57.1	46.3	69.5

4.5 Design of Cyanophage-specific PCR Primers

The main purpose of obtaining sequence data from three cyanophages was to design PCR primers which would amplify cyanophage DNA; specifically, DNA only from cyanophages, but from as many cyanophages as possible. With regard to the sequence data obtained, the primers should amplify DNA only from the three cyanophages.

The DNA "Pileup" alignment of cyanophage and T4 sequences revealed several regions where there is greater conservation amongst cyanophages than between cyanophages and T4 (Fig. 4.7a). Two regions were chosen for the design of PCR primers; the regions thought to be most likely to produce cyanophage-specific primers. These regions had greatest conservation amongst cyanophages, yet with a sufficiently different sequence in T4 to prevent its amplification. These two regions, from which the PCR primers were designed, run from bases 1168-1192 and 1311-1333, according to the sequence from cyanophage strain S-PM2 in Figure 4.7a. The section of the "Pileup" alignment which contains the PCR amplification region, with the primers marked alongside, is shown in Figure 4.9.

Hence, two, potentially cyanophage-specific PCR primers were designed, and named CPS1 and CPS2 (cyanophage-specific). CPS1 is the forward primer and CPS2 is the

reverse primer. PCR amplification using these primers would give a 165 bp product, with an inter-primer region of 118 bases. The primers were made slightly degenerate in order to amplify all the cyanophages. CPS1 has 4-fold degeneracy, and CPS2 has 8-fold degeneracy. The precise sequences of the primers are as follows:

CPS1: 5'-GTAG(T/A)ATTTTCTACATTGA(C/T)GTTGG-3'

CPS2: 5'-GGTA(G/A)CCAGAAATC(C/T)TC(C/A)AGCAT-3'

Melting temperatures, in °C, were calculated as 4(G+C) + 2(A+T) (see Chen and Suttle, 1995a). The melting temperature ranges are: 66-68°C (CPS1); and 64-70°C (CPS2). Hence, the lowest annealing temperatures are 61°C and 59°C for CPS1 and CPS2 respectively. CPS2 is based on the complementary strand for the region 1311-1333, according to cyanophage strain S-PM2; hence, the sequence runs from 1333-1311.

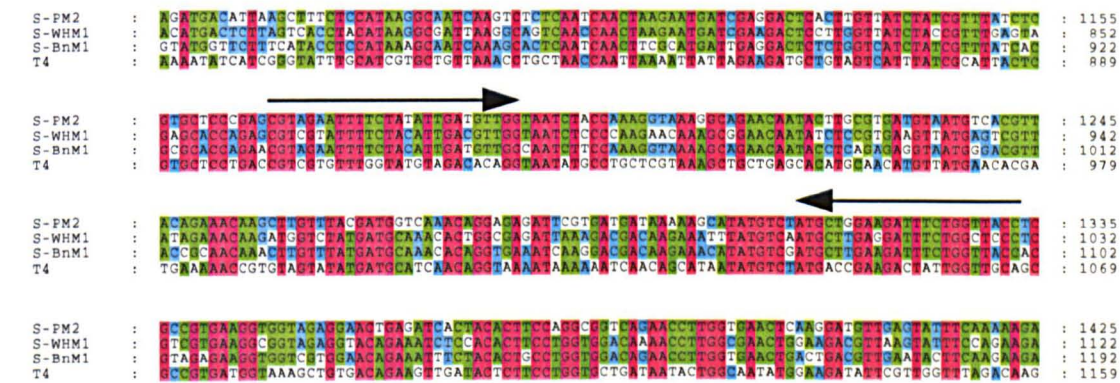


Figure 4.9 Design of cyanophage-specific PCR primers. Section of a DNA "Pileup" alignment, using the software package GCG, of T4 g20 homologues from three cyanophage strains (S-PM2, S-WHM1 and S-BnM1) and T4. Two, potentially cyanophage-specific, PCR primers were designed, based on regions conserved in the cyanophages, but not T4, and are marked alongside (CPS1 and CPS2). Conserved bases are highlighted to four levels.

4.6 Discussion

4.6.1 Isolation of DNA fragments from three cyanophages

An important observation which resulted from the many Southern hybridisation analyses of cyanophage genomes is the presence of just a single DNA fragment from each restricted genome which contains the T4 g20 homologue (unless the homologue was cut in two) (*e.g.* Fig. 4.2). Such an observation was also noted by Wilson (1994). It would therefore seem fairly clear from these studies that there is only a single copy of this T4 g20 homologue in marine cyanophages. This result would be important in the consideration of quantifying cyanophages by PCR.

Another observation from Figure 4.2 is that cyanophage DNA appears to be resistant to digestion from a number of restriction endonucleases. This phenomenon was previously observed by Wilson *et al.* (1993), who suggested the presence of modified bases in cyanophages which had been propagated in the host *Synechococcus* sp. strain WH7803. Ohki and Fujita (1996) observed similar resistance to endonucleases by a cyanophage infecting the marine cyanobacterium *Phormidium persicinum*.

4.6.2 Sequence data and analysis

The "Pileup" alignment of the cyanophage and T4 sequence data reveals limited homology between the T4 g20 homologues of these viruses (Fig. 4.7). It is evident that the three cyanophages are more similar to each other than to T4 (Table 4.1), as would be expected, given that they infect completely different hosts. A similar observation was made with viruses of eukaryotic algae (Chen *et al.*, 1996), in which viruses which infected a particular algal host genus were genetically more similar to each other than to those which infected a different algal host genus. Other studies have also reported capsid proteins which are conserved amongst different families of viruses in the marine environment: *e.g.* viruses of brown algae (Muller *et al.*, 1996;

Sengco *et al.*, 1996); fish iridoviruses (Mao *et al.*, 1997); and fish nodaviruses (Nishizawa *et al.*, 1997).

Table 4.1 reveals the degree of similarity between the virus sequences. However, these figures are an average over the whole open reading frame, and mask regions of much greater similarity, and much greater variability, as can be seen by the "Pileup" alignment (Fig. 4.7); indeed this also shows regions of addition and deletion. In particular, there is a region near the centre of each ORF (residues 256-288 according to the sequence from cyanophage strain S-PM2; Fig. 4.7b), in which 33 consecutive amino acids are 100% conserved amongst the three cyanophages, yet only 58% conserved with T4. It may be that this region, and the extended conserved region, of the peptide have a conserved function amongst the cyanophages (see review by Saier, 1996); a function necessary for all the cyanophages.

The "Pileup" alignment shows marked variability in the length of the ORFs (Fig. 4.7). While all three cyanophage sequences align well at the start of the ORF, they all stop at different positions. In particular, cyanophage strain S-PM2 has a much longer 3' extension than the other cyanophages, and all the cyanophages are extended at the 3' end relative to T4, though the content of their extension is not particularly conserved. It may be that the function of the C-terminus of the peptide is not conserved amongst cyanophages, and is perhaps not as important as the conserved regions for the function of the peptide as a whole. In T4, gp20 is responsible for the initiation of head assembly (Van Driel and Couture, 1978), DNA packaging (Hsiao and Black, 1978) and for binding with the head-tail junction (Coombs and Eiserling, 1977). However, without knowing the precise function of the T4 gp20 homologues in these cyanophages, the function of the C-terminus is uncertain.

It is not surprising to note that the degree of peptide similarity, based on the type of amino acid, is considerably higher than that of peptide identity (Table 4.1) (a common observation, *e.g.* Mao *et al.*, 1997; Monod *et al.*, 1997). If the same peptide is to perform the same function in a similar cyanophage it is to be expected that similarity of residues will be sufficient in certain locations, and that the residues need

not be identical, to enable the peptide to function properly. It is, however, perhaps surprising to note that the degree of DNA identity is fairly similar to peptide identity, amongst cyanophages, and is considerably greater than peptide identity between cyanophages and T4 (Table 4.1). However, this has also been observed elsewhere, for example, in coliphages (Monod *et al.*, 1997). Given the degeneracy of the genetic code one might expect less conservation at the DNA level, since in many cases only the first two bases of the codon need be conserved to encode a specific amino acid. Indeed, this is observed for residues 256-288 (encoded by bases 1107-1205) in cyanophage strain S-PM2 (Fig. 4.7). The greater similarity between viruses at the DNA level than at the peptide level, over the entire ORF, may therefore be a result of slightly different alignments of the sequences. Alternatively, and perhaps more likely, it may be that variable regions, in which the amino acids differ between viruses, coincidentally have one or two bases which are identical between viruses, within a codon.

A recent molecular study (Monod *et al.*, 1997) on the genomes of a group of myoviruses termed pseudo T-even phages revealed a region of limited homology with T4. This region was characterised for the coliphage RB49, and contains genes encoding structural proteins. In particular, partial sequence data revealed the presence of a homologue of T4 g20. Whilst these data are very limited, the homologue exhibits similarity both to T4 g20 and the three cyanophage T4 g20 homologues (Fig. 4.8 and Table 4.2). Cyanophage strain S-WHM1 appears to have an equivalent degree of similarity to RB49 as to T4 (Table 4.2). As might be expected, with similar hosts, the coliphage RB49 sequence is more similar to that of the coliphage T4 than to those of the marine cyanophages (Table 4.2). It has been proposed that this region of the virus genome, which encodes structural proteins, may be a mobile genetic module that can be exchanged as a unit between different viral genomes (Monod *et al.*, 1997). Hence, a similar module may also be present in marine cyanophages. However, the sequence data (*ca.* 300 bp) upstream of the T4 g20 homologue in cyanophage strain S-PM2 showed no significant homology to anything on the database (results not shown). Hence, if a mobile module is involved it is possible that it does not continue upstream of the T4 g20 homologue.

The very fact that marine cyanophages show a degree of similarity with two enteric coliphages (T4 and RB49) is somewhat surprising; their respective hosts are vastly different, both in environment and function, although enteric viruses have frequently been found in coastal, though not oceanic, waters, presumably from sewage pollution (Zobell, 1946; cited by Moebus, 1987; Paul *et al.*, 1997). One theory (Monod *et al.*, 1997) is that, if the T4 g20 homologue is part of a mobile genetic element, it may be passed between viruses via co-infection of the same host. Whilst viruses are generally very host-specific (see review by Borsheim, 1993), rare mutants of T4 have been isolated which infect a completely different, non-enteric bacterial genus, *Aeromonas* (Ackermann *et al.*, 1985; cited by Tetart *et al.*, 1996). Genetic recombination in the gene for the tip of the tail fibre is suggested to allow a great expansion of the host range of T4 (Tetart *et al.*, 1996). It is not inconceivable, therefore, that a mutant T4 could reach coastal waters and infect a *Synechococcus* sp. cell, although the probability would likely be minute. However, the fact that cyanophages containing this homologue seem to be ubiquitous throughout the world's oceans (Chapters 5 and 6 of this study), suggests that this may be an unlikely explanation.

It may simply be that the conserved sequence represents a function of the gp20 which is conserved amongst cyanomyoviruses, T4 and RB49 (see review by Saier, 1996). Such conservation of structure and function, and therefore sequence, are classically observed in 16S rRNA and DNA polymerase, across genera and even phyla (*e.g.* Chen and Suttle, 1996; see review by Fuhrman *et al.*, 1994). The fact that only T4 and RB49, from the entire DNA database, show any similarity with the cyanophages, may simply be a result of the very limited number of bacteriophage sequences available for comparison, especially of phages of the Myoviridae family. A recent search of the database, with "capsid protein" and "bacteriophage" revealed a total of 243 sequences (using Protein Query of Entrez at the National Centre for Biotechnology Information). However, to my knowledge, T4 is the only myovirus, other than P4, which has been entirely sequenced. However, P4 is in a different subgroup from T4 (A1 and A2, respectively; see Ackermann *et al.*, 1995), and showed no significant homology with T4 g20 (results not shown). Hence, it is quite

possible that other myoviruses, in the same subgroup as T4 (A2), possess the T4 gp20 homologue, but that it has not been sequenced. A lack of cross-hybridisation of Myoviridae DNA, from other marine bacteriophages, with the cyanophage probe (Chapter 3 this study) does not contradict this theory, since not even T4 DNA was sufficiently similar to show cross-hybridisation under the conditions used. Hence, it may be that gp20 is conserved, to varying degrees, throughout many phages of the family Myoviridae.

4.6.3 Design of cyanophage-specific primers

Primer design was by visual analysis of the "Pileup" of the four virus sequences (Fig. 4.9). Whilst computer-based techniques are now available, which may be useful for designing even better PCR primers, visual analysis appeared to be sufficient to design primers which proved to be cyanophage-specific (Chapter 5 this study). Whilst molecular characterisation of cyanophage populations should be possible, based on a 165 bp PCR product, a larger product would give more information. However, no other, more suitable sites for primer-design were found. CPS2 has only 8-fold degeneracy, due to the highly conserved sequence. However, Chen and Suttle (1995a) specifically amplified microalgal-virus DNA, using primers which had degeneracies as high as 8192-fold. In this study, degenerate primers were designed initially, in order to ensure that as many cyanophages as possible would be amplified. For the detection of marine cyanophages from the natural environment, non-degenerate primers might well have an undesirable specificity.

Several other studies have also generated virus-specific PCR primers for the marine environment. Brautigam *et al.* (1995) designed non-degenerate PCR primers based on the coat glycoprotein, gp-1 (Klein *et al.*, 1995), of a virus (*EsV*) infecting the marine brown alga, *Ectocarpus siliculosus*. Primers have also been designed to amplify algal-virus DNA, based on the DNA polymerase gene (Chen *et al.*, 1994). These primers were designed from regions in the deduced amino acid sequences which were conserved amongst three microalgal viruses, and were therefore highly

degenerate in order to account for the degeneracy of the genetic code. They have since been used to amplify specifically DNA from viruses which infected three different genera of microalgae (Chen and Suttle, 1995a). PCR primers have been designed from a conserved region of the major capsid protein of fish iridoviruses, and amplified DNA from viruses infecting fish, reptiles and amphibians (Mao *et al.*, 1997). Primers have also been designed from a coat protein gene in fish nodaviruses, and used for phylogenetic analysis of 25 nodavirus isolates (Nishizawa *et al.*, 1997).

In order to design primers which were specific to cyanophages it would have been more desirable to identify conserved regions amongst more than three cyanophages. However, as previously mentioned, these primers proved to be sufficiently specific. Chen *et al.* (1994) designed PCR primers from three algal virus sequences, which later proved to be specific for algal viruses (Chen and Suttle, 1995a). Furthermore, the primers designed by Brautigam *et al.* (1995) were designed from a single *EsV* sequence, but amplified specifically isolates of *EsV* and *EfasV* (*Ectocarpus fasciculatus* virus) collected from different geographic locations (Klebl *et al.*, 1996; Sengco *et al.*, 1996).

Chapter 5:

Development of PCR of Cyanophage DNA

5.1 Introduction

Most of the research into marine cyanophages has been into abundance, morphology and host range (*e.g.* Suttle and Chan, 1993; Waterbury and Valois, 1993; Suttle and Chan, 1994). Relatively little research has been made into molecular studies of marine cyanophages (Wilson *et al.*, 1993; Wilson, 1994; Ohki and Fujita, 1996). Studies into abundance have been based on infection techniques, which are limited by the host strains in laboratory culture, and can take many days. Hence, throughout environmental biology, more and more emphasis is being placed in molecular approaches, such as Southern/Northern analysis, PCR, DGGE, RFLP, *etc.* (*e.g.* Ogunseitan *et al.*, 1992; Muyzer *et al.*, 1993; Scanlan *et al.*, 1996; Teske *et al.*, 1996). These techniques have the ability to detect organisms with a greater sensitivity than traditional microbiological techniques (for review see Fuhrman *et al.*, 1994). Such approaches are now being adopted in other areas of marine microbiology, including analysis of other marine virus systems (for review see Wilson and Mann, 1997). Hence, the main aim of this study was to use such molecular techniques, in particular PCR, to detect and enumerate marine cyanophages.

PCR has been used widely in recent years for the analysis of environmental populations (see references above). PCR has also been used as a method for rapid quantification (for review see Zimmerman and Mannhalter, 1996). This is especially appealing for marine cyanophage systems, since current quantification techniques can take many days. Furthermore, quantification is limited, only counting cyanophages which infect specific host strains used for the experiment. In contrast, PCR should detect most cyanophages, and has potential for giving quantitative results in just a matter of hours. In addition, PCR is able to provide sufficient amounts of DNA to be of use for molecular characterisation of cyanophages. Such characterisation of PCR products is made for example, by RFLP analysis (Ernst *et al.*, 1995; Scanlan *et al.*, 1996), and increasingly by DGGE analysis (*e.g.* Muyzer *et al.*, 1993; Wawer and Muyzer, 1995; Teske *et al.*, 1996). Whilst these techniques

provide more rapid characterisation (a few hours), more complete, yet more lengthy (a few days), characterisation may be made by sequence analysis of the PCR products (*e.g.* Nishizawa *et al.*, 1997).

Once oligonucleotide primers have been designed, PCR conditions must first be optimised before application to environmental samples. Reaction components of the PCR have varying effects upon the final product (for reviews see Giovannoni, 1991; Zimmerman and Mannhalter, 1996), and these must be adjusted to obtain the optimal product concentration and purity. Typical parameters which affect the product include: the number of reaction cycles; MgCl₂ concentration; primer concentration; target DNA concentration; and annealing temperature.

5.2 Aims

The aim of this chapter, having designed putative cyanophage-specific PCR primers (Chapter 4, this study), was to develop PCR of cyanophage DNA in order to be of use for interrogating natural marine populations. First it was necessary to optimise the PCR, then to determine the specificity of the primers, to establish if they would specifically amplify cyanophage DNA. An attempt was made to develop a method for quantifying natural marine cyanophages using competitive PCR (cPCR). In addition, attempts were made to characterise PCR products, amplified from a range of cultured cyanophages, by RFLP, bisbenzimid-PEG separation (an alternative to DGGE) and by sequence analysis. Contents of this chapter are the basis of a manuscript submitted to Applied and Environmental Microbiology (Fuller *et al.*, 1998. In press).

5.3 Results

5.3.1 Optimisation of PCR

PCR conditions (2.8.10.1), using the putative cyanophage-specific primers CPS1 and CPS2 (4.5), were used only as a result of optimisation experiments. The annealing temperature of 55°C (slightly lower than the calculated temperature; 4.5) was chosen to ensure that other cyanophages, with greater mismatch in the primer-binding regions, would still be amplified. Initial conditions differed in that 2.5 µl of 50 mM MgCl₂ and 1 µl of 1 µM of each primer were used, with the reaction proceeding for 30 cycles. Such conditions did result in a PCR product, but not of the maximum concentration possible. Hence optimisation was attempted to improve product yield, quantifying the product by scanning laser densitometry (2.8.10.3) (Fig. 5.1). The parameters which were optimised were: number of cycles; MgCl₂ concentration; and primer concentration, respectively.

Optimisation of PCR conditions (Fig. 5.1; for gels see Append. 2) reveals that conditions for the maximum concentration of product are: 35 cycles; 10 mM MgCl₂; and 200 nM of each primer. Note: it appears that PCR using greater primer concentrations results in more product; however, if the primer concentration is too high then other products (*ca.* 300 bp and *ca.* 400 bp) begin to appear (Append. 2c). So the maximum concentration was used which gave negligible secondary products (40 nM). Hence, these conditions (2.8.10.1), annealing at 55°C, were used throughout the course of this project, unless stated otherwise.

5.3.2 Specificity of PCR primers

To determine the specificity of the primers, CPS1 and CPS2, PCR amplification (2.8.10.1) of DNA from a range of different viruses was attempted (Table 5.1). Some

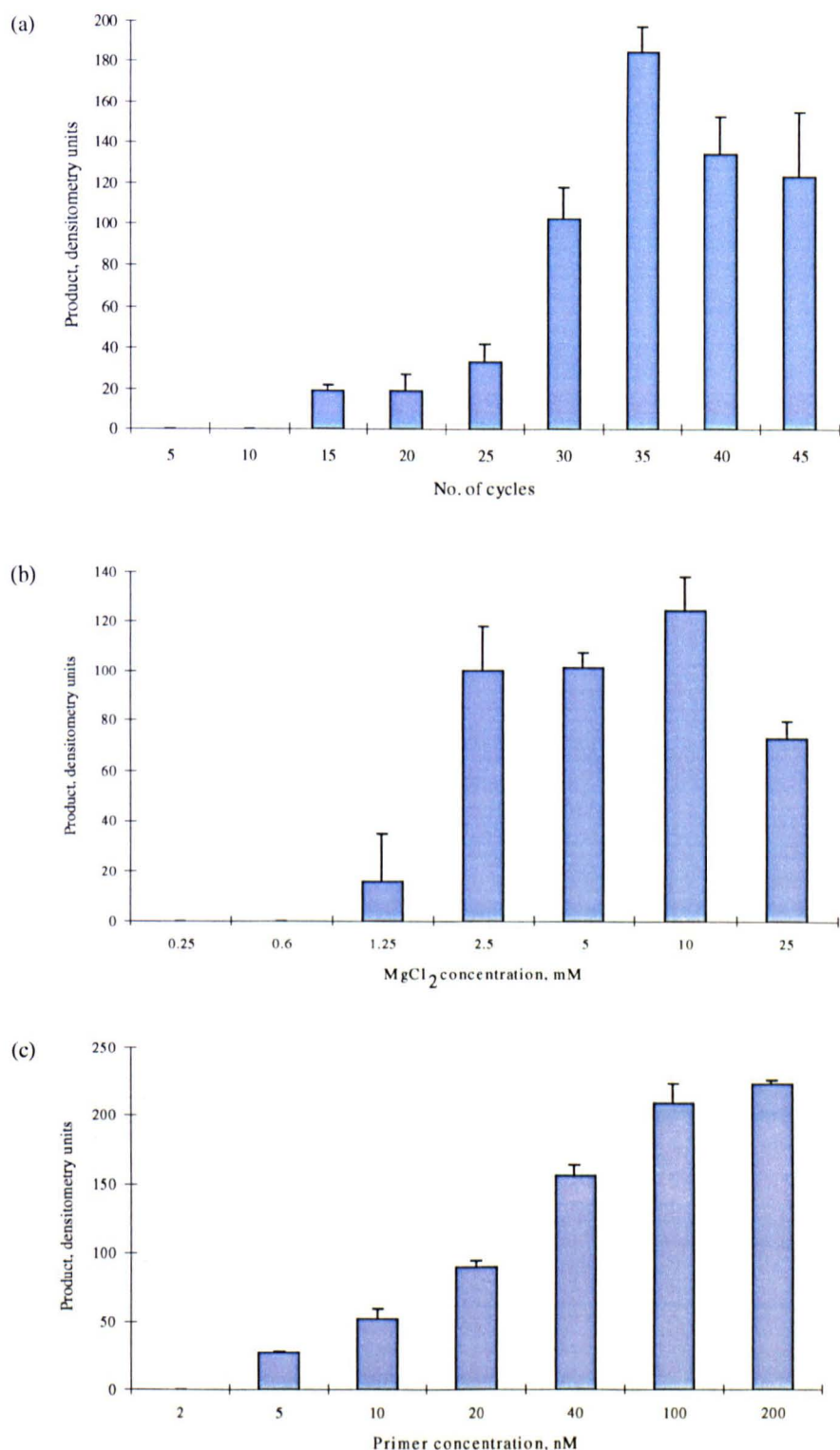


Figure 5.1 Optimisation of PCR conditions for the putative cyanophage-specific primers CPS1 and CPS2. The conditions which are optimised are: (a) cycle number; (b) $MgCl_2$ concentration; and (c) concentration of each primer. For each of the subsequent tests, the previous optimised condition was used. The average of triplicate samples, in relative densitometry units with standard deviation bars, was plotted against the PCR conditions. The initial amount of target DNA was kept at 100 ng per reaction, and the annealing temperature was kept at 55°C.

cyanophages were screened by performing PCR using preparations of their DNA, while for others PCR was performed directly using cyanophage lysate (see later in this Chapter). Many marine cyanophages were screened, of which most were cyanomyoviruses. Four cyanopodoviruses were used as useful controls to determine whether the probes were specific to all cyanophages or just to those in the family Myoviridae, since all three cyanophages from which the primers were designed, and T4, belong to the family Myoviridae.

Various other control viruses were also screened, to check that the primers would not just amplify DNA from any marine bacteriophage, especially those in the family Myoviridae. In addition to marine cyanophages, phages which infect marine heterotrophic bacteria were kindly donated, after many unsuccessful attempts to isolate any (Chapter 3, this study). Other control viruses include those infecting marine phytoplankton and a freshwater cyanophage. The final control viral DNA was T4, bought from Sigma Chemicals. This was expected to produce a negative result due to the specific design of the primers (Fig. 4.9).

The results of the PCR analysis of these viruses are shown in Figure 5.2. A positive result is indicated by the presence of a 165 bp product. In the negative tracks, where no 165 bp product was observed, a faint smaller band representing dimerisation of the primers was observed (not visible in the Figure), and proved to be a useful internal control, demonstrating that the reaction has not been inhibited.

The results shown in Figure 5.2 are tabulated in Table 5.1 to show more clearly which virus DNAs are amplified by the primers, and to compare this with host range, place of isolation and whether DNA from this virus cross-hybridised with the 1 kb *Bam*HI/*Eco*RI DNA probe from S-PM2 (Chapter 3, this study; Wilson, 1994). Cyanophage strains S-MM1 and Φ 2 give very faint products (165 bp), not visible in Figure 5.2. Improved analysis of PCR of cyanophage strains S-BP1, S-BP2 and S-BP3 (not shown) reveals that there is no 165 bp product, or any larger product, for these strains.

The results of the above experiment to determine primer specificity give a very valuable range of specificity, with the primers giving amplification products only for marine cyanomyovirus isolates. This experiment was performed using an annealing temperature of 55°C. Similar PCRs were performed, using annealing temperatures of 50°C and 60°C. These results (Fig. 5.3) show that annealing at 50°C results in many non-specific amplification products; hence, the specificity is too low. However, at 60°C there are no PCR products from many cyanomyovirus isolates; hence, the specificity is too great.

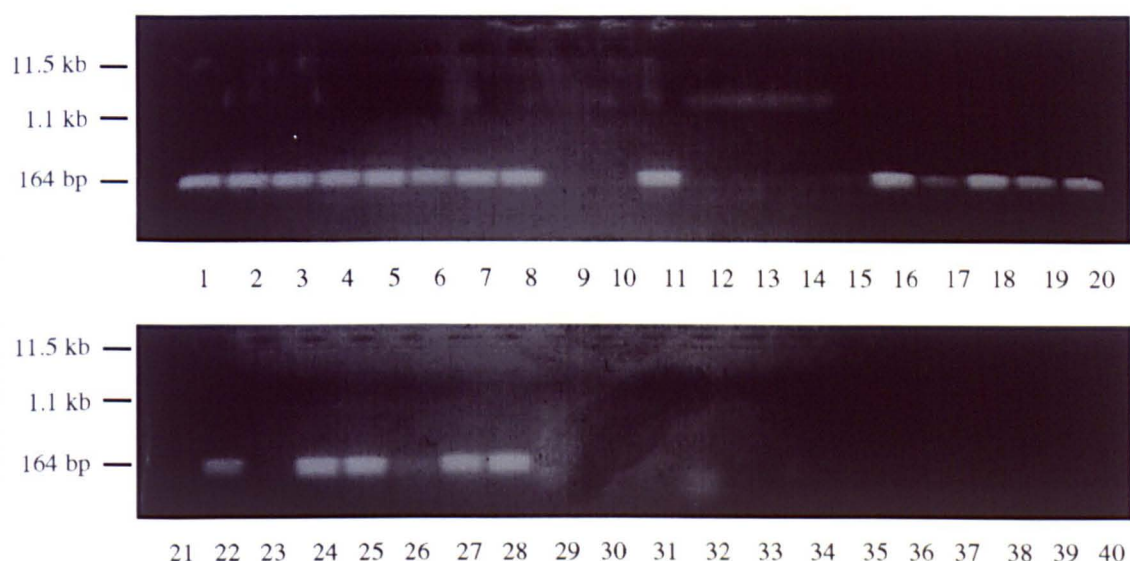
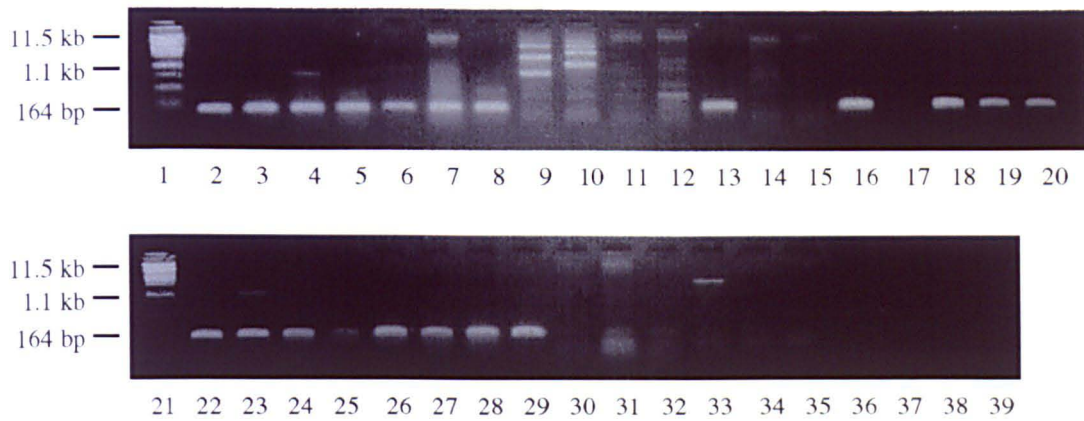


Figure 5.2 PCR amplification of DNA from a range of different viruses, using the putative cyanophage-specific primers, CPS1 and CPS2. The primers were designed from a region in the T4 g20 homologue which was conserved amongst three cyanophages. The resulting reaction mixtures, some containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h. Tracks contained DNA from the following virus strains: (1) S-PM2; (2) S-BnM1; (3) S-WHM1; (4) S-WHM2; (5) S-RSM1; (6) S-RSM2; (7) S-BM1; (8) S-BM3; (9) S-BM4; (10) S-BM5; (11) S-BM6; (12) S-BP1; (13) S-BP2; (14) S-BP3; (15) S-MM1; (16) S-MM2; (17) S-MM3; (18) S-MM4; (19) S-MM5; (20) S-MM7; (21) Φ 2; (22) Φ 9; (23) Φ 12; (24) Φ 14; (25) Φ 33; (26) Φ 34; (27) S-PWM1; (28) S-PWM3; (29) AN-15; (30) PW3a-P1; (31) H2/1; (32) H4/4; (33) H54/1; (34) *PpV*-01; (35) *PpV*-02; (36) *PpV*-04; (37) *PpV*-05; (38) *PpV*-06; (39) *MpV*-Sp1; (40) T4.

Table 5.1 Specificity of the putative cyanophage-specific PCR primers, CPS1 and CPS2. The primers were designed from a region in the T4 g20 homologue which was conserved amongst three cyanophage strains (S-PM2, S-WHM1 and S-BnM1). (a) Virus DNA amplified by CPS1 and CPS2; (b) Virus DNA not amplified by CPS1 and CPS2. Family: M = Myoviridae; P = Podoviridae; S = Siphoviridae; Ph = Phycodnaviridae. Host: WH7803, WH8018, WH8103, WH8012, SYN48 and SNC2 are all phycoerythrin-containing strains of *Synechococcus* sp. and SNC1 is a phycoerythrin-absent strain of *Synechococcus* sp.. Those host strains in parentheses are not infected by the virus strain. The column "CH" shows which viral DNA cross-hybridised with a 1 kb *Bam*HI/*Eco*RI fragment containing part of the T4 g20 homologue from cyanophage strain S-PM2 (those with no + or - were not tested). H2, H4 and H54 are all heterotrophic bacterial strains.

Strain	Family	Host	Place of isolation	CH
(a)				
S-PM2	M	WH7803, WH8012, WH8018. (WH8103)	Plymouth Sound, UK	+
S-BnM1	M	WH7803. (WH8018)	Bergen, Norway	+
S-WHM1	M	WH7803, WH8012, WH8018. (WH8103)	Woods Hole, USA	+
S-WHM2	M	WH7803, WH8012	Woods Hole, USA	
S-RSM1	M	WH7803	Red Sea	+
S-RSM2	M	WH7803	Red Sea	
S-BM1	M	WH7803, WH8012, WH8018. (WH8103)	Bermuda	+
S-BM3	M	WH7803, WH8103. (WH8018)	Sargasso Sea	+
S-BM6	M	WH7803	Sargasso Sea	
S-MM1	M	WH7803	Miami, USA	
S-MM2	M	WH7803	Miami, USA	
S-MM3	M	WH7803	Miami, USA	
S-MM4	M	WH7803	Miami, USA	
S-MM5	M	WH7803	Miami, USA	
S-MM7	M	WH7803	Miami, USA	
Φ2	M	WH7803, WH8018, WH8012. (WH8103)	Sargasso Sea	
Φ9	M	WH7803, WH8018, WH8103, WH8012	Woods Hole	
Φ14	M	WH8103. (WH7803, WH8018)	Gulf Stream	
Φ33	M	WH7803	Gulf Stream	
Φ34	M	WH8103. (WH7803)	Gulf Stream	
S-PWM1	M	WH7803	Gulf of Mexico	
S-PWM3	M	WH7803, SYN48, SNC2, SNC1	Gulf of Mexico	
(b)				
S-BM4	M	WH7803, WH8018. (WH8103)	Bermuda	+
S-BM5	M	WH8018. (WH7803)	Bermuda	+
S-BP1	P	WH8018. (WH7803, WH8103)	Bermuda	-
S-BP2	P	WH8018. (WH7803, WH8103)	Bermuda	-
S-BP3	P	WH7803. (WH8018, WH8103)	Bermuda	-
Φ12	P	WH8018. (WH7803, WH8103)	Gulf Stream	-
AN-15	M	<i>Anabaena</i> sp. PCC7120	Freshwater	
PW3a-P1	M	<i>Vibrio natriegens</i>	Gulf of Mexico	
H2/1	M	H2	North Sea	-
H4/4	P	H4	North Sea	-
H54/1	S	H54	North Sea	-
PpV-01	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	
PpV-02	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	
PpV-04	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	
PpV-05	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	
PpV-06	Ph	<i>Phaeocystis pouchetii</i>	Bergen, Norway	
MpV-Sp1	Ph	<i>Micromonas pusilla</i>	Southern California	
T4	M	<i>Escherichia coli</i>		-

(a)



(b)

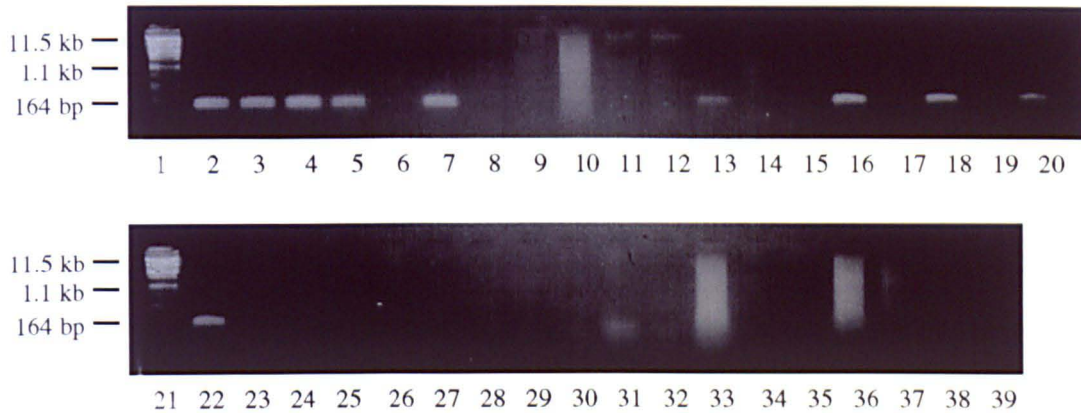


Figure 5.3 PCR amplification at different annealing temperatures of DNA from a range of different viruses. The putative cyanophage-specific primers, CPS1 and CPS2, were designed from a region in the T4 g20 homologue which was conserved amongst three cyanophages. The resulting reaction mixtures, some containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h. Annealing during the PCR was at (a) 50°C; and (b) 60°C. Tracks contained DNA from the following virus strains: (1) λ (*Pst*I); (2) S-WHM1; (3) S-BnM1; (4) S-PM2; (5) S-RSM1; (6) S-RSM2; (7) S-BM1; (8) S-BM3; (9) S-BM4; (10) S-BM5; (11) S-BP1; (12) S-BP2; (13) S-BM6; (14) S-BP3; (15) Φ 12; (16) Φ 14; (17) S-MM1; (18) S-MM2; (19) S-MM3; (20) S-MM4; (21) λ (*Pst*I); (22) S-BnM1; (23) S-MM5; (24) S-MM7; (25) Φ 2; (26) Φ 9; (27) Φ 33; (28) Φ 34; (29) S-WHM2; (30) H2/1; (31) H4/4; (32) H54/1; (33) T4; (34) *PpV*-01; (35) *PpV*-02; (36) *PpV*-04; (37) *PpV*-05; (38) *PpV*-06; (39) blank.

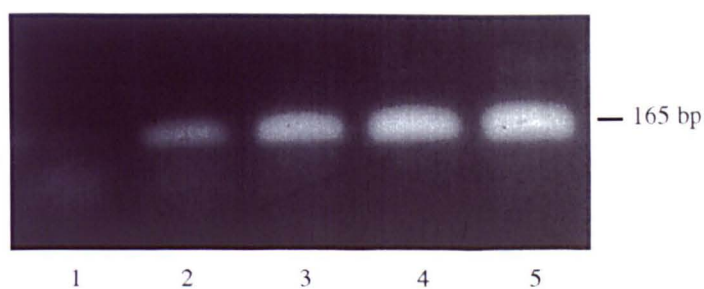
Synechococcus sp. chromosomal DNA might have naturally similar regions of DNA able to bind to the primers, or it may have phage genomes integrated into the chromosome as prophages. To ensure that the PCR products result from amplification of free virus DNA, and not host chromosomal DNA, which might feasibly contaminate a cyanophage lysate stock or DNA preparation, PCR amplification was attempted using both purified chromosomal DNA and whole cells from each of the three *Synechococcus* sp. strains possessed in the laboratory, *i.e.* strains WH7803, WH8018 and WH8103. In addition, two strains of the marine prochlorophyte, *Prochlorococcus* sp. (strains SARG and MED; see Scanlan *et al.*, 1996) were also screened in a similar manner. Chromosomal DNA was kindly donated by Nyree West. The results of this screening of host strains (not shown) revealed that none of the strains resulted in a PCR product, whether using just culture or purified DNA.

5.3.3 Sensitivity of PCR primers

Initial determination of the sensitivity of the PCR primers was made in terms of the target DNA concentration (Fig. 5.4). The concentration of the undiluted DNA was calculated by spectrophotometry (2.8.7). Figure 5.4 illustrates that PCR can give a detectable product from just 100 fg of target DNA. At 100 pg a "plateau" is reached, above which there is no further increase in product.

In order to determine whether the PCR primers would be able to detect cyanophages in the marine environment, at natural concentrations ($0-10^5$ PFU ml⁻¹; Suttle and Chan, 1993), it was necessary to determine the sensitivity of the PCR primers, according to cyanophage concentrations. However, since in a lysate there may be non-viable cyanophages, yet still containing genomes which would be amplified by PCR (see review by Cann, 1993), total cyanophage counts were made, rather than viable counts by plaque assay. This was done by epifluorescence microscopy of lysate dilutions stained with the nucleic acid dye, TOTO-1 (Molecular Probes, Inc.)

(a)



(b)

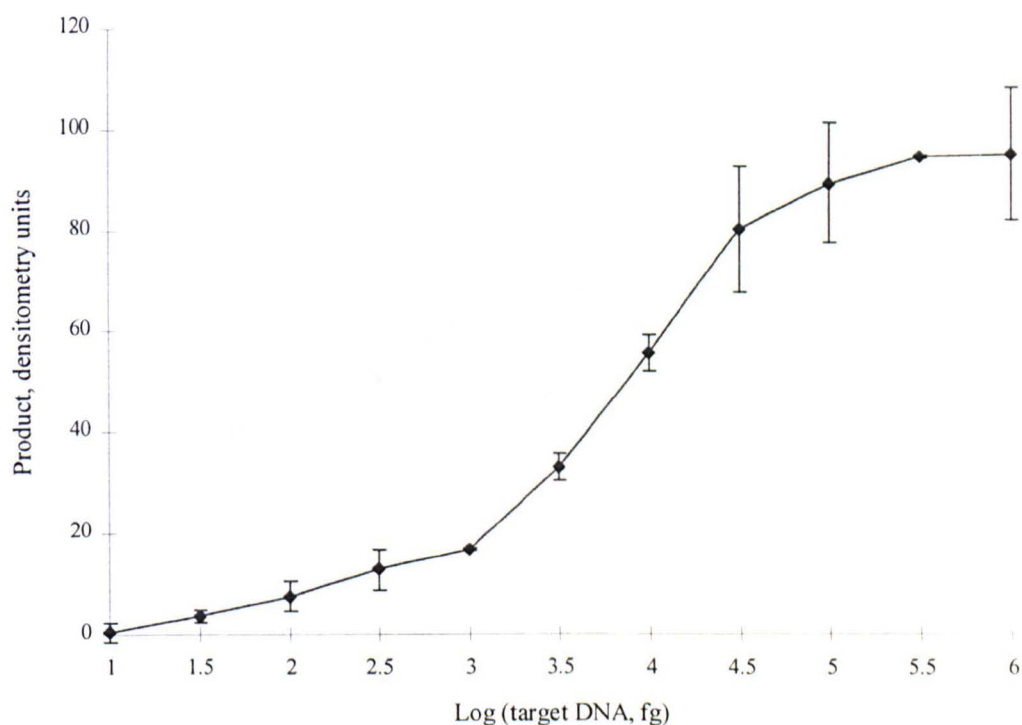


Figure 5.4 Sensitivity of cyanophage-specific PCR primers, CPS1 and CPS2, regarding target DNA concentration. PCR was performed using decimal dilutions of a preparation of cyanophage DNA. The resulting reaction mixtures, containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h, and quantified by scanning laser densitometry (2.8.10.3). (a) Agarose gel showing PCR products. Tracks contain the following amounts of DNA, in fg: (1) 1×10^2 ; (2) 1×10^3 ; (3) 1×10^4 ; (4) 1×10^5 ; (5) 1×10^6 . (b) Quantitative relationship between amount of target DNA and PCR products; each point representing the average of triplicate results, with standard deviation bars.

(2.10). TOTO-1 shows up viruses as tiny, bright yellow dots against a black background (Fig. 5.5). Using cyanophage strain S-WHM1, plaque assays (2.6.1.1) resulted in a titre of 2.1×10^7 PFU ml⁻¹. In contrast, TOTO-1 counts resulted in a titre of 2.3×10^9 virus particles ml⁻¹, *i.e. ca.* 100-fold greater than from plaque assays.

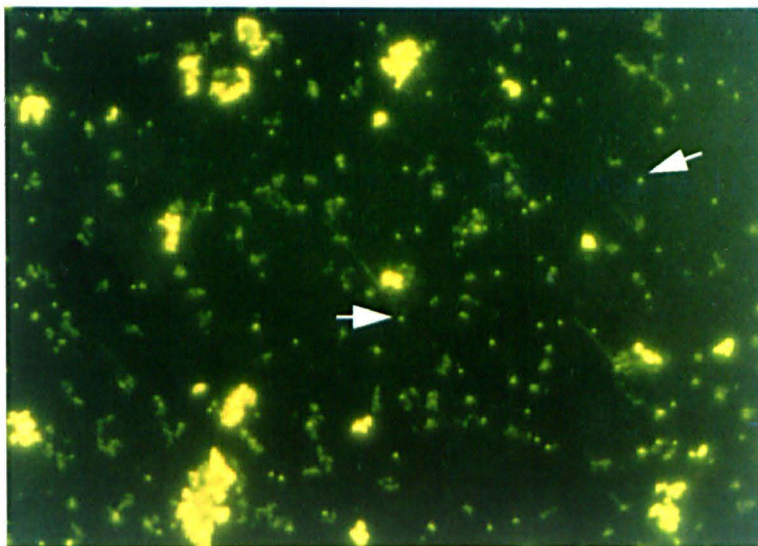


Figure 5.5 Cyanophage lysate (strain S-BnM1), diluted to 10^{-3} and stained with the nucleic acid dye, TOTO-1. The tiny, bright yellow dots, marked with arrows, are individual cyanophage particles. The cyanophages are viewed with a UV epifluorescence microscope, using a blue filter (520 nm).

Initially, cyanophage DNA was purified by phenol extraction before it was used for PCR (2.8.2). However, this method could only detect cyanophages down to an initial concentration of 4×10^4 PFU ml⁻¹, corresponding to *ca.* 4×10^6 cyanophage particles ml⁻¹ (results not shown). Therefore, PCR was attempted directly on cyanophage lysate (similar to Chen *et al.*, 1996). Lysate was always first filtered through a 0.22 μ m pore-size membrane to remove cellular debris. PCR (2.8.10.1) of decimal dilutions of a cyanophage lysate (strain S-BnM1) was compared with total cyanophage counts using TOTO-1 (2.10). The resulting agarose gel (Fig. 5.6) revealed that a PCR product could be obtained from as few as 190 cyanophage particles in 1 μ l of target sample. A similar experiment, using cyanophage strain S-

WHM1, also shows a PCR product from 230 cyanophage particles (corresponding to 2 PFU) in 1 μl (results not shown). This would therefore require a concentration of 10^3 PFU ml^{-1} in a marine sample to enable PCR detection.

In order to detect marine cyanophages at concentrations of less than 10^3 PFU ml^{-1} in the natural environment, it would therefore be necessary to concentrate them. The equivalent of 10-fold concentration can be obtained simply by performing PCR on 10 μl of sample, but in a 100 μl reaction to help minimise salt concentrations. With sufficiently large volumes of seawater initial concentration would be using a tangential flow system (Chapter 6, this study). This should be able to concentrate 100-fold; however, further concentration may be necessary to obtain large quantities of PCR products from environmentally low cyanophage concentrations.

Cyanophages were concentrated by ultracentrifugation (2.11.2) and by PEG precipitation (2.11.3), and their relative concentration efficiencies assessed by TOTO-1 counts (2.10). Ultracentrifugation demonstrated *ca.* 50% concentration efficiency, whilst PEG precipitation was *ca.* 70% efficient. However, in contrast to ultracentrifugation, the concentrate from PEG precipitation was unable to be amplified by PCR (results not shown).

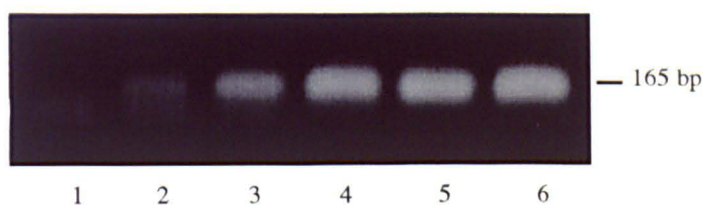


Figure 5.6 Sensitivity of cyanophage-specific PCR primers, CPS1 and CPS2, according to total cyanophage counts. Counts of cyanophage strain S-BnM1 were made by epifluorescence microscopy of TOTO-1-stained lysate. PCR was performed on 1 μl of decimal dilutions of cyanophage lysate. The resulting reaction mixtures, containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h. Tracks contained products from the following numbers of cyanophages: (1) 19; (2) 190; (3) 1.9×10^3 ; (4) 1.9×10^4 ; (5) 1.9×10^5 ; (6) 1.9×10^6 .

Natural seawater samples were obtained from coastal Trinidad and coastal Barbados in January 1997 (according to the method in section 3.2), kindly collected by Lois Vincent-Sealy, and screened by PCR to determine whether cyanophage detection was possible directly from seawater (Fig. 5.7).

The results (Fig. 5.7) show no PCR products for 1 μ l of unconcentrated seawater from coastal Barbados, although a faint product was obtained from 10 μ l.

Ultracentrifugation sufficiently concentrates cyanophages to give a visible PCR product. PCR products are also visible, though very faint, from as little as 1 μ l of unconcentrated coastal Trinidad seawater. Given this result, other samples, collected from different oceanographic regions, were also screened by PCR. Each sample (except that from seawater near Helsinki) had been previously filtered through 0.22 μ m pore-size membranes soon after collection. PCR was then performed in 100 μ l reactions, using 10 μ l of each seawater sample (Fig. 5.8).

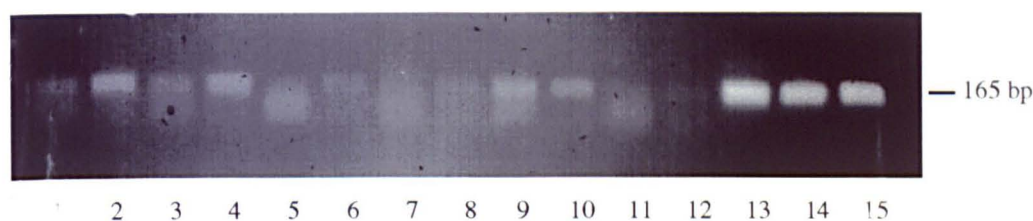


Figure 5.7 PCR of seawater from coastal Trinidad and coastal Barbados. Samples were filtered through a 0.22 μ m pore-size membrane (F), and unfiltered (UF). Filtered seawater from coastal Barbados was also concentrated by ultracentrifugation (2.11.2) from 10 ml to 100 μ l, and used for PCR (C). The supernatant (SN) was also screened by PCR as a control. PCR was using cyanophage-specific primers (CPS1 and CPS2), and performed using both 1 and 10 μ l of sample. Positive controls consisted of cyanophage lysate (strain S-BnM1) added to the ultracentrifuge concentrate, and to filtered coastal Barbados seawater. The resulting reaction mixtures, some containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h. Tracks 1-4 contain seawater from coastal Trinidad; tracks 5-15 contain seawater from coastal Barbados. Tracks contain the following treatments: (1) F, 1 μ l; (2) F, 10 μ l; (3) UF, 1 μ l; (4) UF, 10 μ l; (5) F, 1 μ l; (6) F, 10 μ l; (7) UF, 1 μ l; (8) UF, 10 μ l; (9) C, 1 μ l; (10) C, 10 μ l; (11) SN, 1 μ l; (12) SN, 10 μ l; (13) C + lysate, 1 μ l; (14) C + lysate, 10 μ l; (15) F + lysate, 10 μ l.

Figure 5.8 shows very faintly visible PCR products from seawater off the coasts of: Cape Trafalga, Gambia and Plymouth (concentrated), and extremely faint from

coastal Mauritius. A *Synechococcus* spp. count, made prior to concentration of the Plymouth coast sample by tangential flow filtration, revealed a concentration of 4.9×10^3 cells ml⁻¹. A more visible product resulted from seawater from the coast of Helsinki. All of the samples which were screened were many months (13-22) old, except the sample from the coast of Helsinki, which had been collected 10 days prior to the experiment, by Vicki Cooper. A *Synechococcus* spp. count on this sample revealed a concentration of 1.4×10^4 cells ml⁻¹.

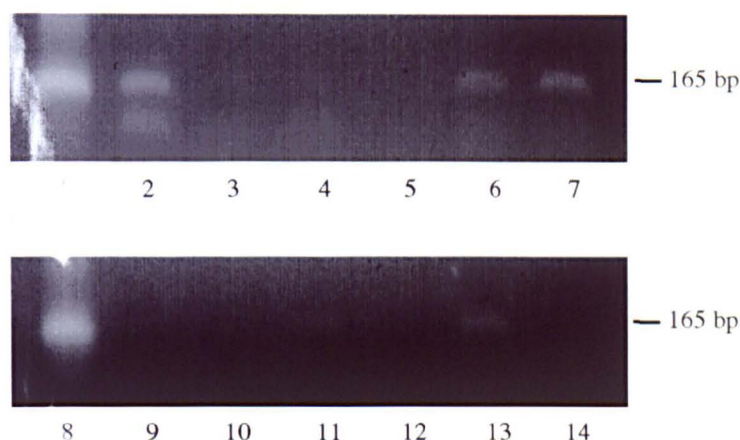


Figure 5.8 PCR of 10 μ l of unconcentrated seawater from different oceanographic locations, using cyanophage-specific primers (CPS1 and CPS2). The sample from the coast of Plymouth was concentrated 100-fold by tangential flow filtration (2.11.1), and PCR was performed on both the concentrated (C) and unconcentrated (UC) sample. Lysate of cyanophage strain S-BnM1, was used as a positive control. The resulting reaction mixtures, some containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h. Tracks 1 and 8 contain cyanophage strain S-BnM1 lysate. The other tracks contain seawater samples off the coasts of: (2) Helsinki; (3) Barbados; (4) Bermuda; (6) Cape Trafalga, SW Spain; (7) Gambia, west Africa; (9) west Gibraltar; (10) Lesbos, Aegean Sea; (11) Mauritius; (12) Plymouth (UC); (13) Plymouth (C); (14) San Pedro, SE Spain. Track 5 contains seawater from Hydrostation S, Sargasso Sea.

5.3.4 Competitive PCR

5.3.4.1 DNase treatment of cyanophage lysate

Quantification of cyanophages was attempted by competitive PCR (cPCR) (1.7.3). In order to calibrate the cPCR technique it was planned to use cyanophage lysate. The lysate titre was established through total counts by staining with TOTO-1. However, it was suspected that a cyanophage lysate may well contain free cyanophage DNA, which may have degraded to varying degrees. Unlike PCR, TOTO-1 counts would not detect free DNA (see Hennes and Suttle, 1995). Hence, PCR would give an artificially high value of cyanophage concentrations. To ensure that PCR only amplified DNA from intact cyanophages, so that a direct comparison could be made with TOTO-1 counts, cyanophage lysates were treated with DNase prior to PCR amplification. However, DNase would also digest the PCR primers, and would therefore need to be denatured before being mixed with them. Optimisation of DNase treatment resulted in the conditions described in section 2.8.10.2.

Having optimised the conditions for DNase treatment of cyanophage lysate, a comparison was then made between treatment and non-treatment with DNase, to ascertain the effect of DNase on a lysate with regard to the subsequent PCR amplification (Fig. 5.9). Figure 5.9 shows the decrease in target DNA due to DNase treatment, resulting in less PCR product. It also shows that cyanophage strain S-WHM1 is more affected by DNase than is cyanophage strain S-BnM1, suggesting that the former lysate contains more free DNA.

5.3.4.2 Design of competitor DNA

For cPCR it was necessary to design a competitor DNA molecule. This needed to be distinguishable from the target PCR product on an agarose gel, since the relative products were to be quantified by scanning laser densitometry of an image of the gel.

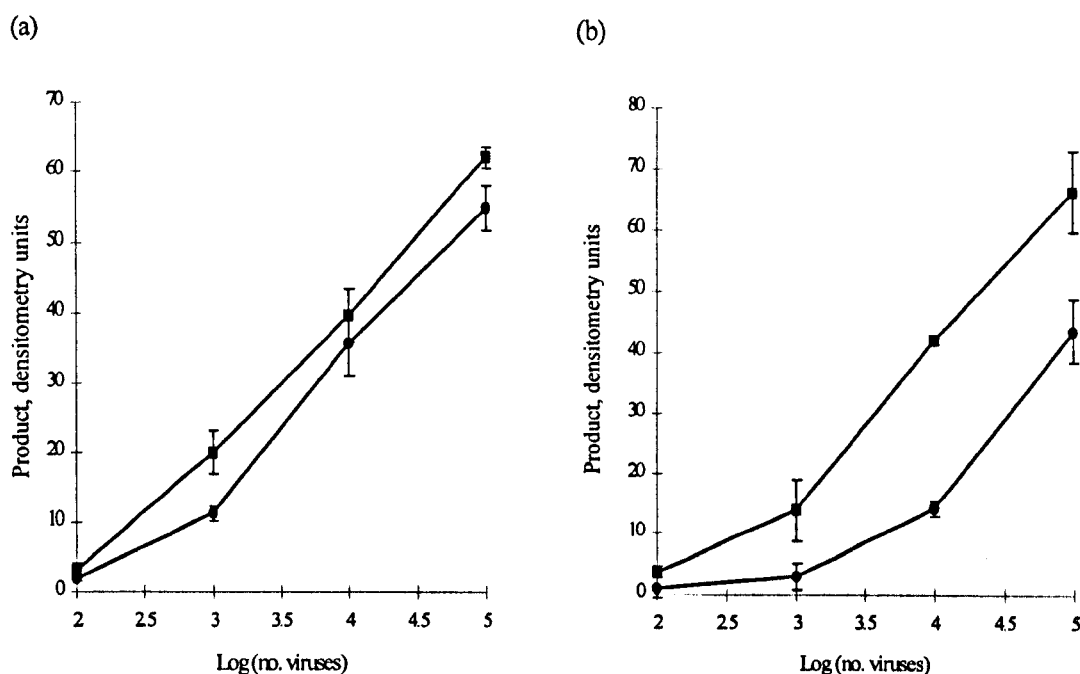


Figure 5.9 PCR of DNase-treated cyanophage lysates. Cyanophage numbers were established by total counts using the nucleic acid stain, TOTO-1. Cyanophage lysates, (a) strain S-BnM1 and (b) strain S-WHM1, were treated with (●) and without (■) DNase (2.8.10.2). PCR products were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h, and quantified by scanning laser densitometry. The mean of triplicate samples is plotted, with standard deviation bars.

Since the target product is already small (165 bp), it was decided to make the competitor product larger. To do this it was necessary to find a restriction endonuclease site, within the region spanned by the PCR primers, in one of the three cyanophage sequences, into which a small piece of DNA could be cloned. Since the three recombinant plasmids (pWHW01, pWHW02 and pWHW04), designed by Wilson (1994), contain the sequenced T4 g20 homologues from each of the three cyanophages, these were used for the design of a competitor. Restriction sites were identified which cut only within the inter-primer region. The most appropriate enzyme was *Eco*NI, which cuts pWHW04, containing part of the homologue from cyanophage strain S-BnM1 (a 1.5 kb *Eco*RI/*Pst*I fragment).

It was then necessary to obtain an appropriate piece of DNA to clone into pWHW04, without any *EcoRI* or *PstI* sites. A suitable piece of DNA was a 193 bp *SphI/PvuII* fragment from pUC19. This 193 bp fragment was purified (2.8.4) and ligated into the *EcoNI* site of pWHW04, after blunting both fragments (2.8.9.3) (Fig. 5.10). A resulting clone containing the enlarged *EcoRI/PstI* insert (*ca.* 1.7 kb) was then identified by PCR (2.8.10.1) and termed pNJF3. PCR of the purified (2.8.4) 1.7 kb *EcoRI/PstI* fragment gave a purer product than did pNJF3. Hence, the 1.7 kb fragment was subsequently used as the competitor, giving a PCR product of 358 bp, distinguishable from the target 165 bp product (Fig. 5.12).

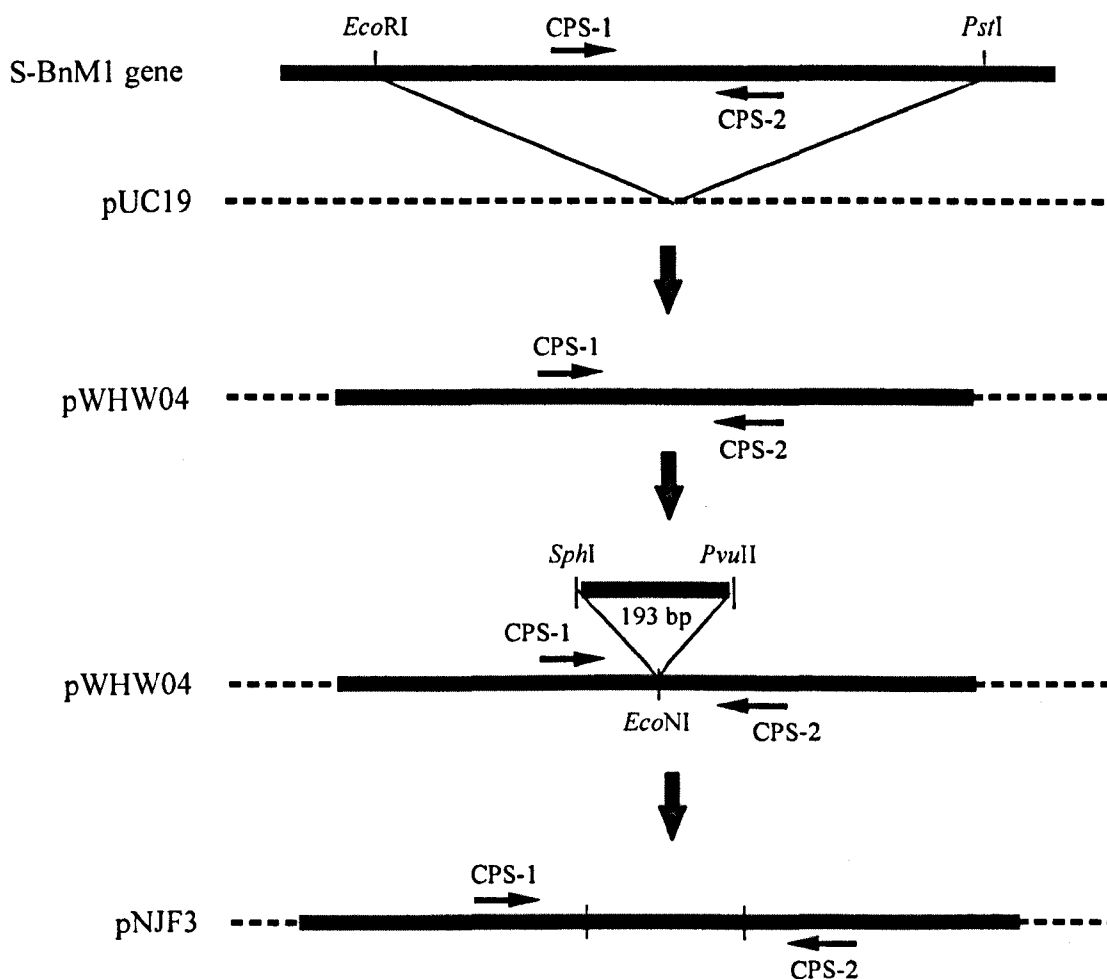


Figure 5.10 Design of an internal standard for competitive PCR using the cyanophage-specific primers CPS1 and CPS2. A 1.5 kb *EcoRI/PstI* fragment, isolated from cyanophage strain S-BnM1, had been cloned into pUC19 (pWHW04; Wilson, 1994). A 193 bp *SphI/PvuII* fragment from pUC19 was subsequently cloned into an *EcoNI* site in pWHW04 to enlarge the region amplified by the primers.

5.3.4.3 Calibration curves

Competitive PCR was based on the technique described by Zachar *et al.* (1993). In order for cPCR to reliably quantify marine cyanophages, it was necessary that the final products were quantified whilst the PCR was still in the exponential phase (see Zachar *et al.*, 1993). Hence, PCR was performed with a similar quantity of cyanophage DNA as might be present in the marine environment (100 pg; compare Figs. 5.4 and 5.6), with the optimised PCR conditions (2.8.10.1), to determine the most appropriate number of cycles for cPCR. The results (Append. 3) showed that 35 cycles was near the end of the exponential phase, and was used for subsequent cPCR.

In order to quantify an unknown sample of cyanophages, it was necessary to draw a calibration curve (Zachar *et al.*, 1993), performing cPCR on known concentrations of cyanophages, with which PCR of unknown samples could be compared. With the mechanics of cPCR, if the competitor was too abundant, relative to the target, then all the product would be from the competitor, with no target product. Hence, it was necessary to find an appropriate concentration of competitor to add to each to each PCR mixture (see review by Zimmerman and Mannhalter, 1996). PCRs with different amounts of competitor (results not shown) revealed an optimum of 2 pg of competitor DNA per reaction. The resulting cPCR is shown in Figure 5.11.

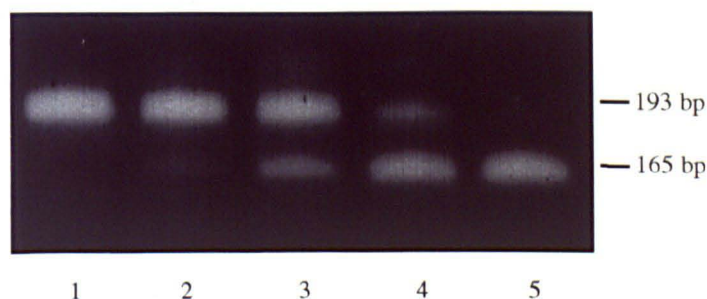


Figure 5.11 Competitive PCR of cyanophage DNA. Each track contains 2 pg of competitor and: (1) 100 fg; (2) 10³ fg; (3) 10⁴ fg; (4) 10⁵ fg; (5) 10⁶ fg of cyanophage strain S-BnM1 DNA, which were each amplified by PCR using primers CPS1 and CPS2. The competitor product is 358 bp, while the target cyanophage (S-BnM1) product is 165 bp. The resulting products were run on a 1.2% (w/v) agarose gel at 60 mA for 1.5 h.

A calibration curve was made, using cyanophage particles, so that accurate quantification of unknown samples of cyanophages could be achieved. However, in an unknown marine sample it is highly unlikely that all the cyanophages will be identical. Hence, for quantification of such a sample, it is necessary that the calibration curves be the same for each cyanophage. To investigate whether this is the case, calibration curves were drawn for three different cyanophages. Competitive PCR was performed with lysates of cyanophage strains S-BnM1, S-MM5 and S-BM3, and the \log_{10} (target product divided by competitor product) was plotted against the \log_{10} (number of cyanophages) (Fig. 5.12). The resulting calibration curves show a log-linear relationship over *ca.* three orders of magnitude of cyanophage numbers.

Figure 5.12 clearly shows that the three calibration curves are not identical, but appear specific for each individual cyanophage, although the gradients of the regressions for cyanophage strains S-MM5 and S-BM3 are not significantly different at the 95% confidence interval (see Append. 4). These curves show 1-2 orders of magnitude differences for the number of cyanophage particles predicted by a given value of \log_{10} (target product / competitor product), depending on the cyanophage used. However, comparison of TOTO-1 counts with PCR products from the cyanophage strains S-MM1 and S-RSM2, revealed that these cyanophages gave barely detectable products, if any, from as many as 10^6 cyanophages (results not shown). In contrast, cyanophage strains S-BnM1 and S-WHM1 gave clearly visible PCR products even from 10^3 or 10^2 cyanophages (5.3.3).

5.3.5 Investigation of cyanophage diversity

Changes in base composition in the inter-primer region of PCR products represents different cyanophage strains. Different ways of analysis of such diversity were investigated, including restriction fragment length polymorphism (RFLP) analysis, bisbenzimidazole separation of PCR products and sequence analysis of PCR products.

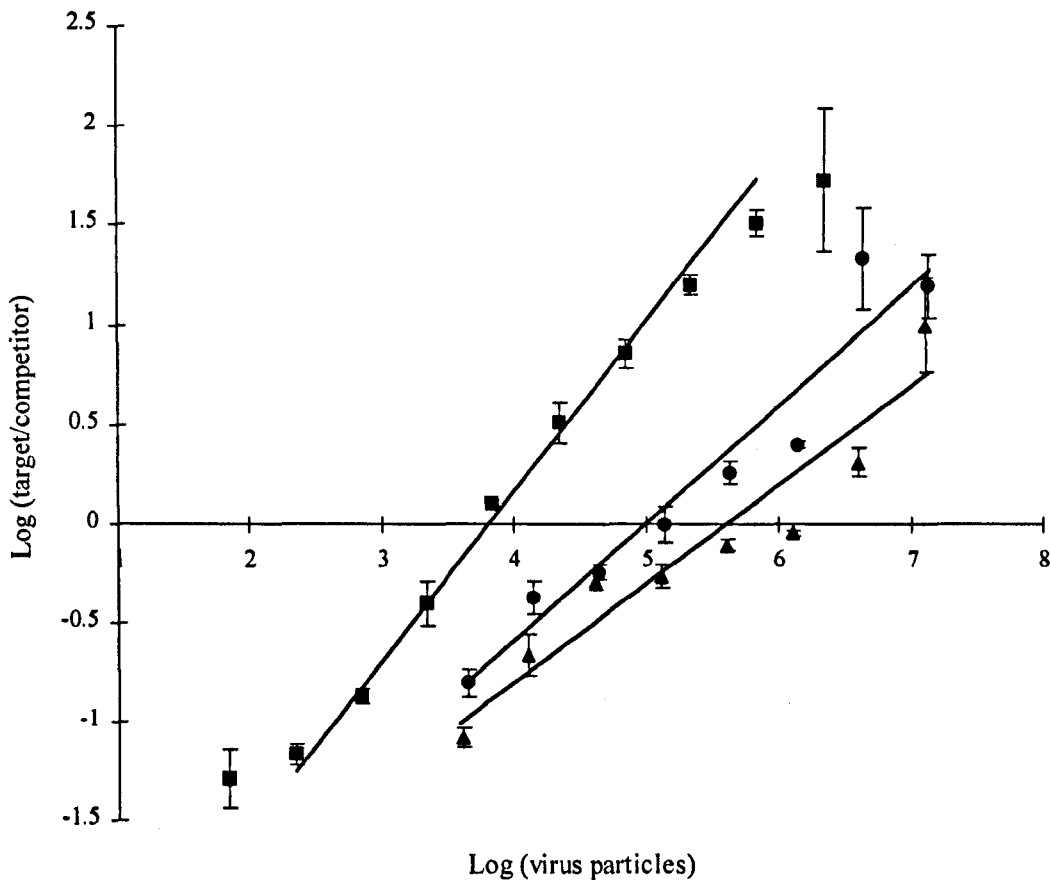


Figure 5.12 Quantitative competitive PCR calibration curves for three cyanophages.

Cyanophage numbers were established by total counts, using the nucleic acid stain, TOTO-1. Lysates were treated with DNase to remove free cyanophage DNA (2.8.10.2) and the primers and 2 pg of competitor DNA were added to each PCR mixture during the "hot start", before addition of *Taq* polymerase. The competitor is based on cyanophage strain S-BnM1 DNA, and contains a 193 bp insert in the region between the primers, CPS1 and CPS2, which were used for the PCR. Products were run on a 1.2% (w/v) agarose gel at 60 mA for 1.5 h, and quantified by scanning laser densitometry. An average of triplicate reactions was plotted, with standard deviation bars, for cyanophage strains S-BnM1 (■), S-MM5 (●) and S-BM3 (▲). A line of "best fit" was drawn for cyanophage strain S-BnM1 by eye. Linear regressions were plotted for cyanophage strains S-MM5 and S-BM3, which are not significantly different at the 95% confidence interval (see Append. 4).

5.3.5.1 RFLP of PCR products

Preliminary characterisation of cyanophage PCR products was by RFLP analysis.

Analysis of the sequence data from the three cyanophages revealed several restriction endonuclease sites in the inter-primer region. *Hinf*I was chosen because it was the

only site of an available and affordable endonuclease. This enzyme should not restrict the PCR product from cyanophage strain S-BnM1; however, it should restrict those from cyanophage strain S-WHM1, giving a 72 and a 93 bp product, and cyanophage strain S-PM2, giving a 118 and a 47 bp product. Restriction by *HinfI* of PCR products from a range of cyanophages (Fig. 5.13) shows three main restriction patterns, which are the same as those demonstrated by the three cyanophages from which sequence data had been obtained.

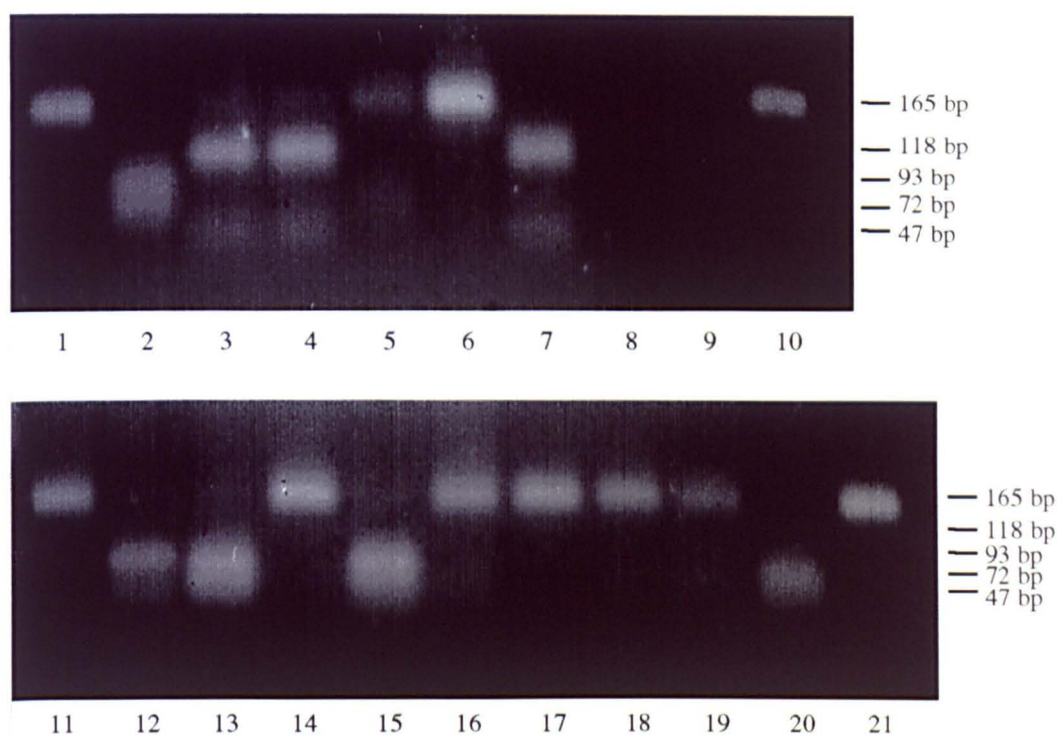


Figure 5.13 RFLP analysis of cyanophage PCR products. PCR products were amplified, using cyanophage-specific primers (CPS1 and CPS2), from a range of marine cyanophage strains: (1) S-BnM1; (2) S-WHM1; (3) S-PM2; (4) S-RSM1; (5) S-RSM2; (6) S-BM1; (7) S-BM3; (8) S-BM4; (9) S-BM5; (10) S-BM6; (11) S-BnM1; (12) Φ 14; (13) S-MM2; (14) S-MM3; (15) S-MM4; (16) S-MM5; (17) S-MM7; (18) Φ 9; (19) Φ 33; (20) Φ 34; (21) Φ 42. 15 μ l of each product was restricted with *HinfI* and run on a 2% (w/v) agarose gel at 50 mA for 2 h.

5.3.5.2 Use of bisbenzimidazole for separation of PCR products

In order to analyse the products resulting from PCR amplification of an environmental sample, it is necessary to separate the different products resulting from different cyanophages present in the sample. Hence, the chemical HA-Yellow (HA-Y; Hans Analytik) was used. This dye, based on bisbenzimidazole with PEG chains attached, binds selectively to adenine and thymine bases in the DNA, retarding DNA migration through an agarose gel to which HA-Y has been added (see Muller *et al.*, 1997). Hence, DNA, such as PCR products, of the same size, but different base composition, can be separated (*e.g.* Wawer *et al.*, 1995).

Conditions for the separation of PCR products using HA-Y were optimised by adjusting the potential difference across the gel, the duration of electrophoresis, and the agarose concentration of the gel. Partial separation, by electrophoresis with HA-Y (2.8.12), of PCR products amplified from lysates of different cyanophages demonstrates that individual clonally purified cyanophages separate out into at least three fragments in the gel (Fig. 5.14). However, it is clear that products from different cyanophages migrate different distances. Since the difference in migration of different products is not very large, a different dye, HA-Red (HA-R), was tried. HA-R binds to the guanine and cytosine bases. The results, however, show minimal separation, far less than when using HA-Y (results not shown).

Multiple bands on the HA-Y gel from PCR products from individual cyanophages may suggest that the cyanophage lysates were not clonally pure, and that the different bands were products from different cyanophages. To test this, cloned PCR products were run on an agarose gel containing HA-Y (2.8.12), and a single band for each cloned product was observed (Fig. 5.15).

A further observation from Figures 5.14 and 5.15 is that, whilst fresh PCR product gives multiple bands in a HA-Y gel, the difference between these bands is generally greater than the difference between cyanophage isolates. A possible reason for this may be that PCR products from an individual cyanophage isolate comprise a mixture

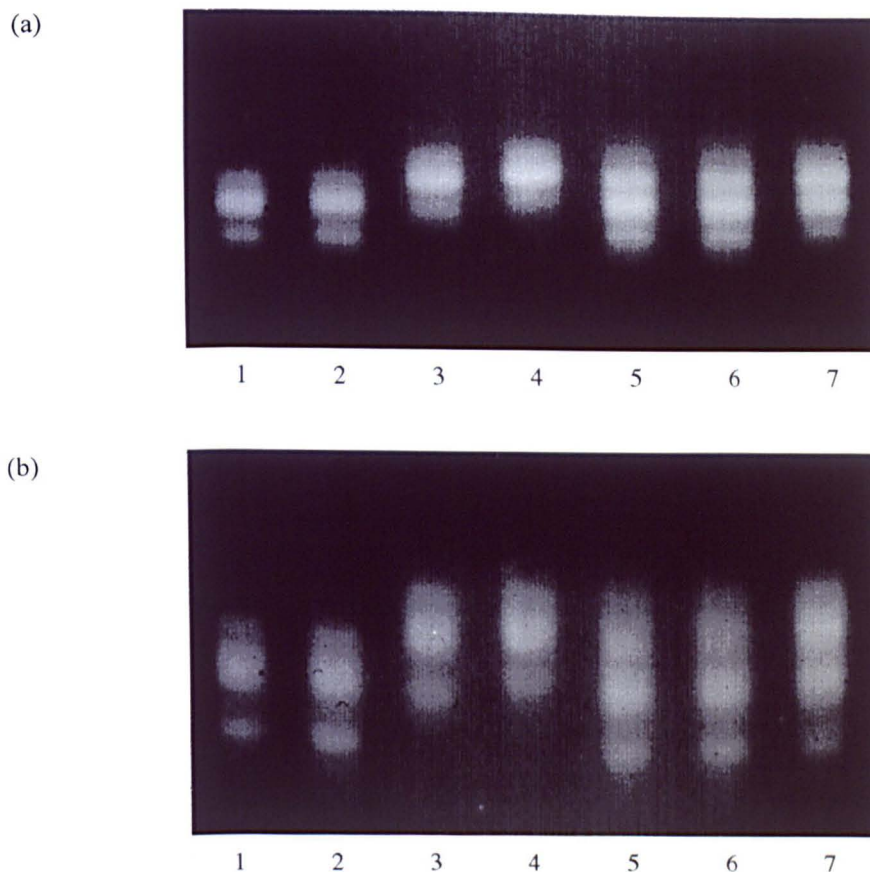


Figure 5.14 Separation of PCR products from different cyanophages, using the dye HA-Yellow (HA-Y; Hans Analytik). PCR products, amplified using the cyanophage-specific primers, CPS1 and CPS2, were run on a 3% (w/v) agarose gel containing 1 unit ml⁻¹ of HA-Y, at 14 V cm⁻¹ for: (a) 30 min; and (b) 60 min. Tracks contain products from cyanophage strains: (1) S-PM2; (2) S-BnM1; (3) S-WHM1; (4) S-RSM1; (5) S-BnM1 + S-WHM1 (co-amplified); (6) S-BnM1 + S-WHM1 + S-RSM1 (co-amplified); (7) S-BnM1 + S-WHM1 + S-RSM1 (amplified individually).

of sequences resulting from degeneracy in the primers, and/or a mixture of products of varying lengths, resulting from the synthesis of primers lacking several bases at the 5' terminus (David Scanlan, personal communication). Hence, new primers were designed, CPS4 and CPS5, which were non-degenerate and had been purified by polyacrylamide gel electrophoresis (PAGE) to ensure that they were all of the one full length. These were based on the consensus sequences of the same regions as CPS1 and CPS2 (Fig. 4.9), and their sequences are as follows:

CPS4: 5' GTAGAATTTTCTACATTGATGTTGG 3'

CPS5: 5' GGTAACCAGAAATCTTCAAGCAT 3'

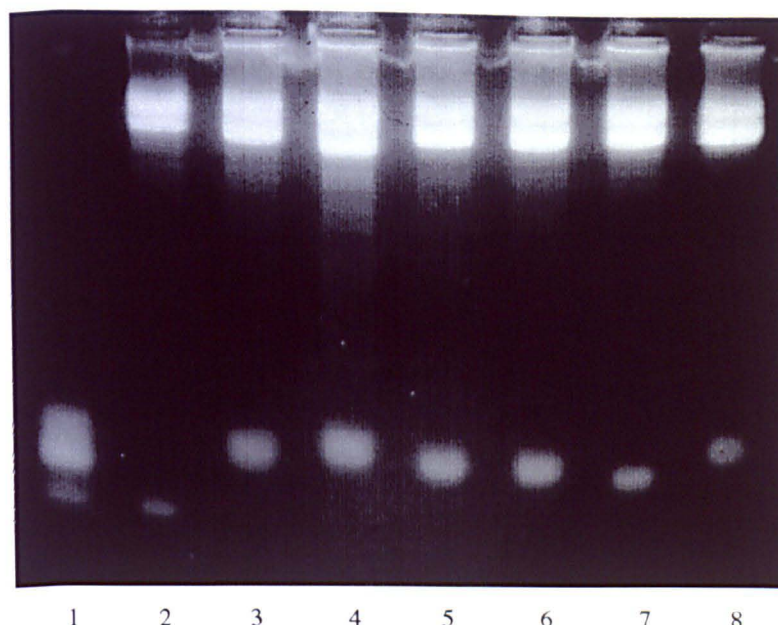


Figure 5.15 Separation of cloned PCR products using the dye HA-Yellow (HA-Y; Hans Analytik). PCR products were amplified using the cyanophage-specific primers, CPS1 and CPS2. Products amplified from cyanophage strain S-PM2 and from different marine samples were cloned into the TA vector, pCR2.1 (2.8.11), then excised by restricting the recombinant plasmid with *Eco*R1. Restriction digests were then run on a 3% (w/v) agarose gel containing 1 unit ml⁻¹ of HA-Y, at 14 V cm⁻¹ for 2 h. A PCR product from cyanophage strain S-PM2 was amplified and run directly on the HA-Y gel as a comparison. Tracks 1 and 2 contain fresh and cloned products, respectively, from cyanophage strain S-PM2. The remaining tracks contain cloned products from the following marine stations (see Chapter 6, this study): (3) 2/1; (4) 2/5; (5) 6/7; (6) 18/1; (7) 18/3; (8) 18/7.

CPS4 and CPS5 were then used to amplify DNA from different cyanophages, and from a marine sample (station 2, 7 m depth; Chapter 6, this study). The resulting PCR products were then run on an HA-Y gel (2.8.12). However, these non-degenerate, length-purified primers still gave multiple bands from individual cyanophage isolates on the HA-Y gel (results not shown). Due to time constraints, no further attempts were made to obtain single bands.

5.3.5.3 Sequencing PCR products

Having gained very limited data from the characterisation of cyanophage PCR products, complete characterisation was performed by sequencing the products (2.8.11). The cloned PCR products, from cyanophage strains S-PM2, S-RSM1, S-

RSM2, S-MM1, S-MM3, S-MM4 and S-MM5, were subsequently sequenced on both strands by automated sequencing. Many clones were screened, and at least two were sequenced for each cyanophage strain, until complete data were obtained for both strands. Cloned PCR products were individually sequenced. Cloning and sequencing were attempted for the PCR products of all the cyanophages present in the laboratory at the time. However, data were only obtained from a limited number of samples. The resulting "Pileup" alignment, using GCG, illustrates the relatively high degree of conservation amongst cyanophages, compared with T4 (Fig. 5.16).

The "Pileup" alignment of PCR product sequences, between the primers, was then analysed using DNA DISTANCE and NEIGHBOUR analyses from PHYLIP version 3.5c (Felsenstein, 1993), in order to produce a dendrogram, illustrating the relative similarity between the virus sequences (Fig. 5.17). This dendrogram illustrates that all of the cyanophages cluster together, distinct from T4, and illustrates the clusters which appear amongst cyanophages.

(a)

```
S-PM2      : TAATCTACCAAAGGTAAAGGCAGAACAATACTTCGCGTATGTAATGT : 47
S-WHM1     : TAATCTCCCCAAGAACAAGCGGAACAATATCTCCGTGAAGTTATGA : 47
S-BnM1     : CAATCTTCCAAAGGTAAAAGCAGAACAATACCTCAGAGAGGTAATGG : 47
S-RSM1     : TAATCTGCCCAAATGAAGGCAGAACAATATCTCCGCGATGTTATGA : 47
S-RSM2     : TAATCTGCCCAAATGAAGGCAGAACAATATCTCCGTGATGTAATGA : 47
S-MM1      : CAATCTTCCGAAGGTAAAGGCAGAACAATACCTTCGTGAAGTCATGA : 47
S-MM3      : TAATCTACCTAAGGTCAAAGCAGAACAATACTTCGCGTATGTCATGT : 47
S-MM4      : CAATCTACCTAAGGTCAAAGGCAGAACAATACTTCGCGTATGTCATGA : 47
S-MM5      : CAATCTACCTAAAGTCAAAGCGGAACAATATCTCCGTGATGTAATGG : 47
T4         : TAATATGCCTGCTCGTAAAGCTGCTGAGCACATGCAACATGTTATGA : 47
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S-PM2      : CACGTTACAGAAACAAGCTTTGTTTACGATGGTCAAACAGGAGAGATT : 94
S-WHM1     : GTCGTTATAGAAACAAGATGGTCTATGATGCAAAACACTGGCGAGATT : 94
S-BnM1     : GACGTTACCECAACAAACTTTGTTTATGATGCAAAACACAGGTGAAATC : 94
S-RSM1     : TGACATATCGTAACAAGCTTTGTGTATGATGCAAAACACTGGAGAGATT : 94
S-RSM2     : TCGGTTATCECAACAAACTTTGTATACGATGCAAAACACAGGAGAGATC : 94
S-MM1      : ATCGTTATCGTTCTAAACTTTGTATATGATGCTAACACGGGTGAGGTT : 94
S-MM3      : CTCGTTATCGTAATAAGCTAGTCTATGATGCACAGACTGGTGAGATG : 94
S-MM4      : GTCGTTATCGCAACAAGCTAGTGTATGACGCAAAACACTGGTGAAATG : 94
S-MM5      : CACGTTATCGTAACAAACTAGTTTACGATGCCAGTACTGGAGAGATC : 94
T4         : ACACGATGAAAAACCGTGTAGTATATGATGCATCAACAGGTAAAATA : 94
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S-PM2      : CGTGATGATAAAAAAGCATATGTCT : 118
S-WHM1     : AAAGACGACAAGAAATTATGTCA : 118
S-BnM1     : AAGGACGACAAGAAACAATGTCTG : 118
S-RSM1     : CGTGATGATAAAAAATACATGGCA : 118
S-RSM2     : CGTGATGACAAAAAGTACATGGCG : 118
S-MM1      : AGAGACGATAAGAAGTTCATGTCT : 118
S-MM3      : CGTGATGACAAAAAGCACATGAGT : 118
S-MM4      : CGTGATGACAAAAAGCATATGAGT : 118
S-MM5      : CGTGATGACAAAAAGCATATGAGT : 118
T4         : AAAAAACAACAGCATAATATGTCT : 118
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Figure 5.16 "Pileup" alignment of the sequences of PCR products amplified from different cyanophages. Products were amplified using the cyanophage-specific primers, CPS1 and CPS2. Alignments were made, using the software package GCG, of: (a) the DNA sequences and; (b) the amino acid sequences, of the cyanophage amplification products and the equivalent region in T4. Sequence data which had previously been obtained from cyanophage strains S-WHM1 and S-BnM1 (Chapter 4 this study) are also aligned. Sequence data only between the primers are shown. These primers amplify a conserved region within a homologue of T4 g20. Conserved bases and residues are highlighted to four levels. Continued overleaf.

(b)

S-PM2	:	NLPKVKAEQYL RDVMSRYRNKL VYD GQTGEIR DOKKHMS	:	39
S-WHM1	:	NLPKVKAEQYL REVMSRYRNKM VYD ANTGEIK DOKKHMS	:	39
S-BnM1	:	NLPKVKAEQYL REVMGRYRNKL VYD ANTGEIK DOKKHMS	:	39
S-RSM1	:	NLPKVKAEQYL RDVMMRYRNKL VYD ANTGEIR DOKKYMA	:	39
S-RSM2	:	NLPKVKAEQYL RDVMMRYRNKL VYD ANTGEIR DOKKYMA	:	39
S-MM1	:	NLPKVKAEQYL REVMNRYRSKL VYD ANTGEVR DOKKHMS	:	39
S-MM3	:	NLPKVKAEQYL RDVMSRYRNKL VYD AQTGEMR DOKKHMS	:	39
S-MM4	:	NLPKVKAEQYL RDVMSRYRNKL VYD ANTGEMR DOKKHMS	:	39
S-MM5	:	NLPKVKAEQYL RDVMARYRNKL VYD STGEIR DOKKHMS	:	39
T4	:	NMPARKAAEHMQHVMNTMKNR VVYD STGKIKNQQHMS	:	39

Figure 5.16 (continued) "Pileup" alignment of the sequences of PCR products amplified from different cyanophages. Products were amplified using the cyanophage-specific primers, CPS1 and CPS2. Alignments were made, using the software package GCG, of: (a) the DNA sequences and; (b) the amino acid sequences, of the cyanophage amplification products and the equivalent region in T4. Sequence data which had previously been obtained from cyanophage strains S-WHM1 and S-BnM1 (Chapter 4 this study) are also aligned. Sequence data only between the primers are shown. These primers amplify a conserved region within a homologue of T4 g20. Conserved bases and residues are highlighted to four levels.

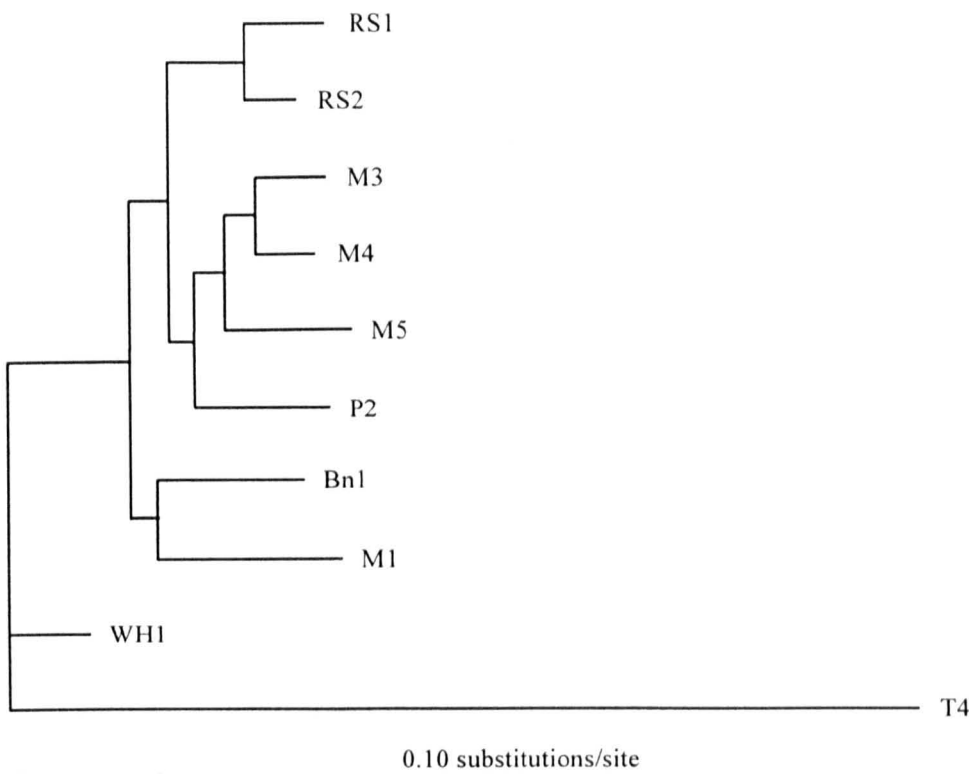


Figure 5.17 Dendrogram of PCR products amplified from different cyanophages using the cyanophage-specific primers, CPS1 and CPS2, and the equivalent region in T4. The dendrogram was constructed using DNA DISTANCE and NEIGHBOUR analyses from PHYLIP version 3.5c (Felsenstein, 1993) of a GCG DNA "Pileup" alignment. Only the sequence data between the primers were used. These primers amplify a conserved region within a homologue of T4 g20. Cyanophage strains are abbreviated (e.g. Bn1 instead of S-BnM1).

5.4 Discussion

5.4.1 Specificity of PCR primers

The results of the experiment to determine the specificity of the putative cyanophage-specific PCR primers, CPS1 and CPS2, summarised in Table 5.1, reveal that, under the conditions used, annealing at 55°C, the primers amplify only marine cyanophages belonging to the family Myoviridae, *i.e.* cyanomyoviruses. However, two cyanomyovirus strains, S-BM4 and S-BM5, gave very small amounts of products of *ca.* 800 bp and 1.1 kb, respectively, compared with the expected product size of 165 bp (not visible in Fig. 5.2). None of the other cyanophages (both marine and freshwater), bacteriophages or algal virus isolates gave PCR products (Fig. 5.2). Cyanophage strains S-BP1, S-BP2 and S-BP3 appear to give large clear PCR products (Fig. 5.2); however, these were an artefact of this reaction, and were not observed at any other time. More clear gels (not shown) clearly show no products for these cyanophages.

Table 5.1 also reveals that the PCR primers amplify DNA from cyanomyovirus isolates regardless of their oceanographic region of isolation; indeed cyanophages from as far apart as the Gulf of Mexico (cyanophage strain S-PWM1) and the Red Sea (cyanophage strain S-RSM1), in different oceans, gave PCR products. Furthermore, the PCR primers amplify cyanophages which infect different strains of *Synechococcus* sp..

Table 5.1 also compares the results of the PCR with those of Southern hybridisation with the 1 kb *Bam*HI/*Eco*RI fragment from cyanophage strain S-PM2, containing the region from which the PCR primers were designed. The results are very similar, as expected. Of the viruses screened for Southern hybridisation, PCR products were only amplified from those whose DNA cross-hybridised with the 1 kb probe. However, whilst most of the viruses which did not give a PCR product did not show cross-hybridisation with the probe, there were two exceptions. The cyanomyovirus

strains S-BM4 and S-BM5 cross-hybridised with the probe yet gave no PCR products of the expected size, although small quantities of unexpectedly large products were amplified.

These results suggest that the primers, CPS1 and CPS2, are indeed specific for cyanophages, since no non-cyanophages were detected (Table 5.1). Although a limited selection of viruses was screened, it would appear that these primers, whilst detecting viruses of some prokaryotic phytoplankton, will not detect viruses of eukaryotic phytoplankton (*MpV*-Sp1 and *PpVs*; Table 5.1). In addition, the primers appear not to detect bacteriophages infecting marine heterotrophic bacteria, even phages of the family Myoviridae (strains H2/1 and PW3a-P1; Table 5.1). This is not surprising, since a recent survey of protein sequence identities from tailed phages revealed that sequence similarities were generally weak, indicating extensive diversity amongst these phages (Ackermann *et al.*, 1995). These results suggest that the primers, when applied to marine samples, would only detect cyanophages, and no other types of virus, using the same PCR conditions, annealing at 55°C.

Since the primers detected none of the cyanopodovirus isolates, yet all of the cyanomyovirus isolates (Table 5.1), it would appear that the primers are specific to cyanomyoviruses. However, it is noticeable that, of all the marine cyanophages which have been isolated, the vast majority belong to the family Myoviridae (*e.g.* Waterbury and Valois, 1993). Hence, it is expected that the primers would detect the majority of culturable cyanophages in a marine sample, and possibly many unculturable cyanophages as well. None of the cyanophages isolated in this laboratory belong to the family Siphoviridae, and very few cyanosiphoviruses have been isolated (Suttle and Chan, 1993). This may either reflect a general scarcity of cyanosiphoviruses, or their relative inability to be isolated on the host strains currently in use, due to a more limited host range (Suttle and Chan, 1993).

Whilst all the cyanomyovirus isolates were detected by the primers, it is surprising that two cyanophage strains, S-BM4 and S-BM5, gave unexpectedly large products (not visible in Fig. 5.2). It is perhaps also surprising that these two cyanophages

should give products of different sizes from each other, given that their restriction patterns are almost identical (Fig. 3.2). According to morphological studies (Tables 3.1 and 3.2) these seem to be typical Myoviridae isolates, and their DNA cross-hybridised with the 1 kb probe from cyanophage strain S-PM2, containing part of the T4 g20 homologue. Yet it is noticeable that cross-hybridisation was relatively weak, in comparison with cyanophage strain S-BM1 (Fig. 3.2). This may suggest that the PCR products resulted from non-specific binding; yet even at a lower annealing temperature no product of the expected size was amplified (Fig. 5.3a). However, at this lower temperature T4 gave an unexpectedly large PCR product, supporting the hypothesis that the products for cyanophage strains S-BM4 and S-BM5 resulted from non-specific binding of the primers. However, it is sufficient to note that the primers gave products of the expected size for the majority of marine cyanomyovirus isolates, and would therefore be valuable for analysing marine cyanophage populations.

Whilst the specificity of the PCR primers is sufficiently high to detect only cyanomyoviruses, it is sufficiently broad for them to detect cyanophages from every oceanographic region from which cyanophages were isolated. The T4 g20 homologue would appear to be sufficiently conserved to enable PCR detection of cyanophages isolated from different oceans; the Atlantic and the Indian (Table 5.1). Indeed, a small amount of product was amplified from water collected from Mauritius, in the Indian Ocean (Fig. 5.8). Whilst no cyanophages were obtained from the Pacific Ocean, it is expected that the primers would detect such cyanophages. Hence, it is expected that these primers would detect cyanophages from any marine sample, regardless of where, in the world's oceans, it was collected, provided that there were sufficient cyanophages for detection. PCR, based on a gene encoding a capsid protein in *EsV* (Klein *et al.*, 1995), a virus infecting a marine brown alga, has been used to detect similar viruses from algae on the coasts of all the world's oceans and continents (Klebl *et al.*, 1996; Sengco *et al.*, 1996). Similarly, a study using DNA hybridisation showed genetic similarity amongst all 60 vibriophages isolated from distinct oceans (Atlantic and Pacific) (Kellogg *et al.*, 1995).

The specificity of the primers is also sufficiently broad to detect cyanophages which infect different host strains of *Synechococcus* sp.. For example, cyanophage strain S-PM2 infects *Synechococcus* sp. strains WH7803 and WH8018 but not strain WH8103, and, conversely, cyanophage strain Φ 14 infects *Synechococcus* sp. strain WH8103 but not strains WH7803 or WH8018, yet the primers detect both of these cyanophages (Table 5.1). In addition, the primers detect cyanophage strain S-PWM3, which infects the phycoerythrin-absent, coastal *Synechococcus* sp. strain SNC1. This cyanophage also infects phycoerythrin-containing strains, *e.g.* *Synechococcus* sp. strain WH7803 (Suttle and Chan, 1993). Hence, this result broadens the specificity range of the primers even further, so that they may even detect cyanophages of phycoerythrin-absent *Synechococcus* sp. strains, which are generally more coastal strains (Waterbury *et al.*, 1986).

These results are valuable, since there may be many cyanophages which cannot be isolated and cultured in the laboratory, simply because they will not infect the host strains available in the laboratory. These primers may well detect cyanophages which infect many marine *Synechococcus* sp. strains which are not available in the laboratory. Since those strains available in axenic culture are probably a tiny fraction of the total number of strains in the marine environment (*e.g.* Fuhrman *et al.*, 1993a; Palenik, 1994), this therefore makes PCR an extremely valuable tool for analysing marine cyanophage populations, vastly expanding the range of cyanophage detection obtainable by traditional microbiological techniques, such as plaque assay.

Whilst the primers detect marine cyanomyovirus isolates, regardless of the host *Synechococcus* sp. strain, they do not amplify DNA from the freshwater cyanomyovirus, AN-15. This result suggests that the primers are specific to marine cyanomyoviruses. However, it cannot be certain whether AN-15 is not detected because it is a freshwater cyanophage, or because it infects a different cyanobacterial genus, *Anabaena*. To determine which reason is correct, it would be necessary to screen a cyanomyovirus which infects a freshwater *Synechococcus* sp. strain (*e.g.* Kim and Choi, 1994). However, a PCR product was obtained from the seawater sample from off the coast of Helsinki (Fig. 5.8), which would have been closer to

freshwater than the salinities found typically in the marine environment. Hence, the primers may be able to detect freshwater cyanophages of *Synechococcus* spp..

Whilst conclusions can be made as to the specificity of the PCR primers, it is acknowledged that the range of controls used to determine the specificity is limited. With regard to marine cyanophages of different families, this probably reflects their natural abundance, or at least their tendency to being isolated. However, to be more certain of the specificity, it would be necessary to screen additional cyanophages, of the families Podoviridae and Siphoviridae, which infect marine *Synechococcus* spp.. To be more certain that the primers are not just myovirus-specific it would be necessary to screen more myoviruses which infect marine heterotrophic bacteria, in particular, of those bacteria which are more common in marine waters, since it is not known how widespread are the bacteria H2, H4 and H54.

Whilst the primers have been termed "cyanophage-specific", they have only been shown to be specific for myovirus cyanophages which infect cyanobacteria of the genus *Synechococcus*. There are, however, other marine cyanobacterial genera, perhaps the most ecologically important being *Prochlorococcus* (Liu *et al.*, 1995). However, to date, no cyanophages have yet been isolated which infect *Prochlorococcus* spp.. Cyanophages which infect *Prochlorococcus* spp. would be a valuable control for the PCR primers, since it is probable that they would be abundant in the marine environment, due to the abundance of their host. Cyanophages have recently been isolated which infect the marine cyanobacterium *Trichodesmium* sp. (Ohki, 1997); these would also be useful controls for the cyanophage-specific primers. It may be that the primers detect cyanomyoviruses which infect other marine cyanobacterial genera; however, the fact that they do not detect AN-15, which infects the freshwater cyanobacterium, *Anabaena* sp., may suggest that the primers are specific to cyanophages of *Synechococcus* spp..

In the light of the results of Chen and Suttle (1995a), described above, it may be possible to detect cyanophages which infect different cyanobacterial genera by designing PCR primers based on a more conserved gene, such as DNA polymerase.

Indeed, preliminary work by Wilson (1994) produced a degenerate DNA probe based on a highly conserved region of DNA polymerase, and observed cyanophage DNA fragments which cross-hybridised to the probe; however, the specificity range of this probe was not investigated.

The results of adjusting the PCR annealing temperature (Fig. 5.3) illustrate that an annealing temperature of 50°C is too low for the detection of only cyanophages, since many other larger products, besides the 165 bp product, resulted. Furthermore, products were obtained from viruses of marine heterotrophic bacteria and from T4. Conversely, an annealing temperature of 60°C is too high, reducing the specificity too greatly, so that many marine cyanomyovirus isolates did not give amplification products. Hence, it would appear that 55°C is the most appropriate of the temperatures attempted. It may have been possible to further refine the annealing temperature, by adjusting it in single degree steps. However, 55°C is sufficient for the specificity required, and annealing at 50°C does not result in 165 bp PCR products from any more cyanomyovirus isolates.

The result of screening host cyanobacterial DNA with the PCR primers (results not shown; 5.3.2) proves that the 165 bp products are amplified from free cyanophage DNA, and not from prophages integrated in those host chromosomes, nor from otherwise similar regions in the host chromosome. Until recently no temperate marine cyanophages had been isolated. However, a temperate cyanophage infecting the marine cyanobacterium *Phormidium persicinum* was recently isolated by Ohki and Fujita (1995; 1996), and a temperate cyanophage has very recently been reported for a marine *Synechococcus* sp. strain (Sode *et al.*, 1997). Southern analysis of chromosomal DNA from cyanophage-infected *Synechococcus* sp. suggested that cyanophage DNA was present with the host chromosome; however, the evidence for lysogeny was not convincing (Wilson *et al.*, 1996).

5.4.2 Sensitivity of PCR primers

The results of initial sensitivity experiments (5.3.3) revealed that PCR would detect down to $100 \text{ fg } \mu\text{l}^{-1}$ of cyanophage DNA (Fig. 5.4), and down to 10^4 PFU ml^{-1} if PCR was performed on DNA preparations of lysate dilutions (results not shown). Comparison of plaque assays with TOTO-1 counts revealed about 100-fold greater counts using the nucleic acid stain TOTO-1 (results not shown; 5.3.3). TOTO-1 is one of a series of nucleic acid dyes with a particularly high fluorescence once bound, but with a very low background fluorescence, and is therefore preferable to the traditional nucleic acid dye, DAPI (Hirons *et al.*, 1994; see Hennes and Suttle, 1995). Furthermore, Fuhrman *et al.* (1993b) showed that counting by DAPI-staining may miss small, dimly-staining viruses. The difference in counts may partly be explained by cyanophages aggregating together, as was occasionally observed during TOTO-1 counts (results not shown). Such aggregations are resolvable for counting the individual cyanophages by TOTO-1 counts, but would result in a single plaque from a plaque assay. Such aggregations may have been caused by fragments of cell debris passing through the $0.22 \text{ } \mu\text{m}$ pore-size filter (3.2), or by Brownian coagulation (Grant, 1994).

The 100-fold difference in counts may be partly due to defective, non-infective cyanophages being produced in a lysate (for review see Cann, 1993), yet still containing a genome to be detected by TOTO-1 staining, and by PCR. Hennes and Suttle (1995) used the similar dye, YO-PRO-1 to stain viruses in seawater samples, and observed that DNase treatment did not reduce the virus counts. They concluded that the fluorescence resulted from virus particles, and was not the result of nucleic acids associated with the surface of particles (Hennes and Suttle, 1995). However, Maruyama *et al.* (1993) suggested that DNA adsorbed to ultramicro particles in seawater may be resistant to DNase digestion.

The ability to obtain PCR products directly using cyanophage lysate (Fig. 5.6) meant that it was not necessary to perform DNA preparations on the lysate, which might

otherwise have posed a greater limitation on the sensitivity of the PCR, and hindered the accurate quantification of marine cyanophages. It is likely that thermal cycling, in particular the "hot start" at 94°C, breaks open cyanophage particles, thus releasing the DNA to be amplified by the *Taq* polymerase (also discussed by Sobsey, 1993). PCR has proved successful for amplifying DNA from individual cells and whole organisms, without prior preparation of the DNA (*e.g.* Gussow and Clackson, 1989; McEwan and Wheeler, 1995; Grevelding *et al.*, 1996), and has been performed directly using virus particles from seawater by Chen *et al.* (1996). Chen *et al.* (1996) observed an increased yield when they preheated and cooled their samples before PCR. Such results were not obtained in this study by similar treatment (results not shown); however, sufficient yield was obtained without such pre-treatment (Fig. 5.6). Therefore, it should be possible to amplify cyanophage DNA by PCR directly on seawater samples, without the time-consuming and loss-incurring need for DNA preparation.

PCR of lysates can detect as few as 190 cyanophage particles from 1 µl (Fig. 5.6), corresponding to 10³ PFU ml⁻¹ (5.3.3). A similar detection limit was observed for HIV-1 DNA, using ethidium bromide staining (Zammatteo *et al.*, 1995). Similarly, HAVs, enteroviruses and rotaviruses have been detected by PCR of as few as 1-100 virus particles, corresponding to 0.05-0.3 PFU (see review by Sobsey, 1993), demonstrating a similar discrepancy between virus particles and PFU as was observed in this study (5.3.3).

According to Suttle and Chan (1993; 1994), cyanophage concentrations are commonly at least 10³ PFU ml⁻¹ in temperate and tropical surface waters. Indeed, these values may be under-estimates, since only a few host strains were used (Suttle and Chan, 1993; 1994). Certainly, if seawater samples were concentrated 100-1000-fold, which can easily be done by tangential flow filtration (Giovannoni *et al.*, 1990b), then it ought to be possible to detect cyanophages in most natural surface concentrations by PCR. Indeed, there may well be sufficient cyanophages in natural surface waters to be detected from as little as 1 µl of unconcentrated water (Fig. 5.7).

Additional methods of cyanophage concentration were investigated, including ultracentrifugation (2.11.2), PEG precipitation (2.11.3) and ZnCl_2 precipitation (2.11.4). ZnCl_2 precipitation, whilst being able to concentrate viruses (Santos, 1991), was not suitable for subsequent PCR (results not shown). PEG precipitation gave similar results (5.3.3). However, ultracentrifugation proved successful in concentrating cyanophages for PCR (Fig. 5.7); although, as observed in 5.3.3, small viruses may not be recovered with 100% efficiency (Borsheim *et al.*, 1990; Hara *et al.*, 1991). Ultracentrifugation has long been used to concentrate viruses from marine samples (*e.g.* Bergh *et al.*, 1989). Previous studies have demonstrated that this was also the preferential method for concentration of viruses from polluted waters (Puig *et al.*, 1994). Results (not shown), suggest that PEG and ZnCl_2 precipitation also concentrate an inhibitor of the PCR, possibly salts from seawater.

Different techniques have been described which have shown greater sensitivity than those used in this study. A bioluminescence technique, based on luciferin-luciferase, has permitted detection of as few as 5 HIV-1 DNA copies, and has shown a 10-fold greater sensitivity than chemiluminescent techniques, *e.g.* using peroxidase (Zammatteo *et al.*, 1995). However, this technique would not be suitable for application to environmental systems, comprising unknown target sequences. Antigen-capture PCR has been used to detect hepatitis A virus (*e.g.* Graff *et al.*, 1993). This may be a suitable method for environmental systems. Nested, or semi-nested PCR has commonly been used as a means of confirmation of PCR products (*e.g.* Chen and Suttle, 1995b), and can also be used to improve sensitivity (*e.g.* Zhang and Martineau, 1996). Furthermore, semi-nested PCR has been used to overcome PCR inhibitors (*e.g.* Miyamoto *et al.*, 1997), and may be useful in overcoming elevated salt concentrations which may result from concentrating marine virus samples (see above). However, the amplification product from CPS1 and CPS2 is already small for obtaining characterisation data, and nested PCR would give an even smaller product. The use of fluorescent primers, coupled with detection by an automated sequencer, has allowed PCR detection of as few as five copies of viral DNA (Thiery *et al.*, 1996).

The results in Figure 5.7 also demonstrate that the sensitivity of the PCR using cyanophage-specific primers, CPS1 and CPS2, is such that it is capable of giving visible PCR products from just 1 µl of unconcentrated seawater, as long as there are sufficient cyanophages present. This result coincides with conclusions made above, regarding the potential sensitivity of the PCR, in that 10^3 PFU ml⁻¹ could be detected. Unfortunately, no *Synechococcus* spp. counts were made from the samples, so it is not known what the likely concentration of cyanophages would be; however, from sensitivity experiments (Fig. 5.6), the concentration in the Trinidad seawater sample is probably at least 10^3 PFU ml⁻¹. Previous PCR assays, of marine microalgal viruses, has required prior concentration of the viruses from seawater (Chen *et al.*, 1996), possibly because of their low natural concentrations (Cottrell and Suttle, 1991). There appears to be no difference in the relative amounts of PCR product from both the filtered and unfiltered samples, suggesting that the products were amplified from particles smaller than 0.22 µm in diameter, and therefore probably resulted from whole cyanophage particles rather than from lysogenised *Synechococcus* spp..

Subsequent PCR using 10 µl of seawater, from several different samples (Fig. 5.8), showed again that cyanophages could be detected by PCR from such a small amount of unconcentrated seawater. The fact that many samples did not give a product, and that most of those which were amplified were faint products, is not surprising given that the samples had been collected 13-22 months prior to PCR. Although the samples were filtered and stored in the dark at 4°C, it is still possible that considerable cyanophage loss may have occurred in that period. Whilst phage lysates have been stored for up to 7 years without much loss in titre (Moebus, 1987; Wilson *et al.*, 1993), phages in seawater suffer inactivation despite such efforts as filtration and cold storage in the dark (Noble and Fuhrman, 1997). It is possible that cyanophages could have adsorbed to the sides of the plastic bottles used for collection of the seawater (see Proctor and Fuhrman, 1992). Unfortunately, no *Synechococcus* spp. counts were made from these samples when first collected, so no estimate can be given as to cyanophage concentrations.

It is important to note that these seawater samples (except the sample from the Helsinki coast) were screened by plaque assay, a few days after collection, and none of them produced any plaques (Chapter 3, this study); yet some of them have given PCR products many months after collection. These results clearly demonstrate the power of PCR over traditional microbiological techniques in detecting cyanophages. No plaques were obtained either because cyanophage concentrations were too low (<10 PFU ml⁻¹), or the cyanophages present were not able to infect the host strain used (*Synechococcus* sp. WH7803). PCR would appear to be able to surmount these problems. Furthermore, even with a concentration step, PCR provides a method of cyanophage detection which is far more rapid (*ca.* 5 h) than traditional infection techniques (*ca.* 5 days). The rapidity and simplicity of the PCR also allows shipboard detection of cyanophages, so that real-time data can be obtained during a scientific cruise.

The fact that the sample from the Helsinki coast resulted in a PCR product (Fig. 5.7) is not surprising, given the *Synechococcus* spp. titre of 10^4 ml⁻¹ (5.3.3). Suttle and Chan (1994) have shown that cyanophage concentrations of 10^5 PFU ml⁻¹ may accompany such host concentrations, which would be easily detectable by PCR using 10 μ l of such seawater.

A main limitation with PCR is that it would not be able to distinguish between viable and non-viable cyanophages, unless the cyanophage was non-viable because it lacked DNA. Another limitation is that PCR would not be able to detect certain bacteriocins. Bacteriocins include phages with empty head and tail assemblies, and are therefore undetectable by PCR, but are capable of lysing cells (see review by Bradley, 1967). Examples of bacteriocins have been found in seawater (Jiang and Paul, 1994), and similar bacteriocin-like particles have been observed in cyanophage lysates in this study (Fig. 3.1a; the three headless cyanophages). Hence, a proportion of cyanobacterial lytic agents may go undetected by PCR.

5.4.3 Competitive PCR

DNase treatment of cyanophage lysates was proven to be necessary before accurate cyanophage quantification could be made by PCR (Fig. 5.9). Dissolved DNA (D-DNA) concentrations in the marine environment can reach $15 \mu\text{g litre}^{-1}$ (Boehme *et al.*, 1993), and viruses can contribute as much as 96% of D-DNA (Weinbauer *et al.*, 1995). However, the importance of free viral DNA in the oceans is unknown. Free cyanophage DNA in a lysate could have made a substantial contribution towards the amount of resultant PCR product. The fact that DNase appeared to have different effects, quantitatively, upon the lysates of cyanophage strains S-BnM1 and S-WHM1, suggests that these two cyanophages may produce different amounts of free DNA in a lysate, *i.e.* the amount of free DNA may not be the same for all cyanophages. The use of intact virus particles for cPCR should not be a problem, since even crude cell lysates can be used with the same reproducibility as purified DNA (see review by Zimmerman and Mannhalter, 1996).

The competitor DNA molecule for cPCR was based on the T4 g20 homologue from cyanophage strain S-BnM1, with a 193 bp insertion into the 165 bp amplification region. Whilst many cPCR experiments use a competitor of a very similar size to the target (*e.g.* Diviacco *et al.*, 1992; Hammerle *et al.*, 1996; Thiery *et al.*, 1996), the size differences in this study are not unreasonable for cPCR. Zachar *et al.* (1993) performed cPCR with a 222 bp competitor, whilst the target DNA gave a 429 bp product. The large size difference facilitates separation and quantification of the products, and also reduces the formation of heteroduplexes, which might otherwise interfere with quantification (see review by Zimmerman and Mannhalter, 1996). However, in order to obtain better results for cPCR it may be preferable to design a competitor molecule with a smaller insert. This would help the competitor to be amplified at a more similar rate to the target, in which case the PCR would not have to be confined to the exponential phase for quantification (see review by Zimmerman and Mannhalter, 1996). Such products could be distinguished, for example, by running the products on a high resolution polyacrylamide gel, and quantifying by

densitometry (*e.g.* Diviacco *et al.*, 1992), or by an automated sequencer if they had been fluorescently labelled (*e.g.* Cammarota *et al.*, 1996).

The method chosen for quantifying the relative amounts of target and competitor PCR products was by scanning laser densitometry of products separated by agarose gel electrophoresis, similar to the method described by Zachar *et al.* (1993).

Densitometry was chosen as it is safe, cheap, quick and easy. An alternative would be to label the products with ^{32}P , excise the products from the gel and quantify them by scintillation counting (*e.g.* Lee *et al.*, 1996); however, this would be more time-consuming and involve the use of hazardous radioactive chemicals. Another, more time-consuming, expensive and complicated method would be to label the PCR products with a fluorescent primer and quantify them using an automated sequencer, running the products on a denaturing acrylamide gel (*e.g.* Thiery *et al.*, 1996), as described earlier.

In order for cPCR to work properly, it is preferable that the target and competitor DNAs be amplified with similar efficiencies (see reviews by Siebert and Larrick, 1992; Zimmerman and Mannhalter, 1996), unless the PCR is restricted to the exponential phase (as discussed by Zachar *et al.*, 1993). Yet even restricted to the exponential phase, the ratio of the two amplification efficiencies should remain constant for cPCR (Zachar *et al.*, 1993). Whilst the relative amplification efficiencies were not compared, the results (Fig. 5.12) show that it is possible to obtain a calibration curve from cyanophage lysate, obtaining a log-linear relationship over *ca.* three orders of magnitude of cyanophage numbers. The type cPCR, by Zachar *et al.* (1993), achieved linearity over four orders of magnitude. Using the same technique, Lee *et al.* (1996) achieved linearity over three orders of magnitude. In contrast, using non-competitive PCR, Romanowski *et al.* (1993) only obtained linearity over two orders of magnitude.

Samples of unknown cyanophage quantity could then be amplified with the same amount of competitor (2 pg) as was used for the calibration curve, and the result compared with the calibration curve to give a value for the original number of

cyanophages. It would therefore appear possible to quantify cyanophages rapidly by cPCR, presuming that the identity of the cyanophage is known and that a calibration curve can be drawn for it (see below).

Since cPCR of three different cyanophages resulted in three different calibration curves (Fig. 5.12), it would appear not to be possible to accurately quantify a sample of unknown cyanophage strain, or a sample of mixed strains, such as would be found in a marine sample. The three calibration curves differed by a maximum initial cyanophage estimation of two orders of magnitude. Attempts were made to reduce this difference by the addition of 5% (w/v) acetamide to the reaction mixture (after Reysenbach *et al.*, 1992). However, this resulted in products which could not be quantified. The difference in calibration curves may result from using degenerate primers (see below). Without the use of non-degenerate primers it is expected that other cyanophages may well produce considerably different calibration curves, rendering quantification of an unknown sample completely impossible. Evidence for this is that the cyanophage strains S-MM1 and S-RSM2 produced virtually no PCR product, even from up to 10^6 cyanophage particles (results not shown; 5.3.3), whereas both cyanophage strains S-BnM1 and S-WHM1 gave clearly visible products from *ca.* 200 cyanophages (5.3.3; Fig. 5.6).

The results of the different calibration curves, and vastly different amount of PCR product for the same number of different cyanophages (5.3.3), may be a result of differing efficiencies with which the primers bind to the target cyanophage DNA, giving differential amplification. Such differences were observed in Figure 5.3, comparing with Figure 5.2, which shows that many, though not all, cyanophages which gave PCR products when annealing at 55°C, failed to give any products when annealing at 60°C.

Therefore, in order to obtain calibration curves which are the same for all cyanomyovirus isolates, and which can therefore be used to quantify cyanophages in marine samples of unknown composition, it may be necessary to design new, non-degenerate PCR primers based on regions which are even more conserved, so that

there is little, or no, mismatching of the primers to different cyanophage DNAs. To find such regions it will be necessary to obtain sequence data of the g20 homologue from more cyanophages. If the primer sequences were much more similar, if not identical, to all marine cyanomyovirus sequences, this may help to reduce or eliminate the problem of differential amplification (see reviews by Giovannoni, 1991; Zimmerman and Mannhalter, 1996).

5.4.4 Investigation of cyanophage diversity

Initial attempts to characterise cyanophage PCR products were made by RFLP and HA-Y analysis since these methods are simple and rapid, giving results in 2-3 h (*e.g.* Wawer *et al.*, 1995; Jackson *et al.*, 1996; Chang *et al.*, 1997). When restricting the PCR products of several different cyanophages with *Hinf*I, it appeared that there were only three restriction patterns (Fig. 5.13). These patterns were the same as those for the three cyanophages which were initially sequenced. Since some cyanophages possess no *Hinf*I sites within this PCR amplification region, it may be preferable to perform RFLP analysis with a different enzyme, which is capable of cutting all cyanophage products. Restriction endonucleases which cut the products from all three of the sequenced cyanophages include *Cje*PI and *Tth*111II; however, these were unavailable. Comparison of Figure 5.13 with Table 5.1 suggests that, using *Hinf*I, there appears to be no correlation between restriction pattern and place of isolation. In addition, there is no correlation between restriction pattern and host strain specificity.

Separation of PCR products by DGGE is a technique which is increasingly being used to analyse environmental samples (Muyzer *et al.*, 1993; Wawer and Muyzer, 1995; Teske *et al.*, 1996). However, initial attempts in our laboratory to develop DGGE using the primers CPS1 and CPS2, with a GC-clamp attached to CPS2, proved unsuccessful; hence, the alternative method, using HA-Y, was adopted. To date, this method has not been used very widely (*e.g.* Wawer *et al.*, 1995; Kristensen

and Borresen-Dale, 1997). However, HA-Y has recently been used successfully to separate PCR products which differ by as little as a single base change (Muller *et al.*, 1997).

The results of HA-Y separation of PCR products show multiple bands for a single, clonally pure cyanophage lysate (Fig. 5.14). The reason for this is unknown; however, possibilities are discussed below. The fact that PCR products which had been cloned into pCR2.1 and excised, resulted in single bands when run on an HA-Y gel (Fig. 5.15), suggests that the multiple bands were an artefact of the PCR, rather than of the HA-Y. Hence an aliquot of PCR product must have a mixture of different products which are separated on an HA-Y gel. Yet the mixture appears to be selective, since there are only three main bands on the gel, and not a smear. An obvious explanation might be that there were three different cyanophages in the lysate. However, this is unlikely, given that the cyanophages were plaque-purified three times (3.2). It is also unlikely that each cyanophage lysate should consist of exactly three different cyanophage strains. In addition, the patterns of the three bands all appear to be the same for each cyanophage lysate (Fig. 5.14). Multiple bands were also obtained by Kristensen and Borresen-Dale (1997) following HA-Y analysis of PCR products from a single target DNA species, though they made no comment regarding this.

It is possible that the multiple bands in the HA-Y gel result from using degenerate primers, or primers of slightly different lengths, such that there is an amplification bias towards three main species of product, even from a single cyanophage strain. However, this appears not to be the case, given that multiple bands were obtained even when performing PCR with non-degenerate, PAGE-purified primers (CPS4 and CPS5) (5.3.5.2; results not shown). Whilst Kristensen and Borresen-Dale (1997) obtained multiple bands with products amplified with non-degenerate primers, their primers were not reported to have been purified by any means. Negative controls for PCR, in which no product was amplified when no target was added, either using CPS1 and CPS2 or CPS4 and CPS5 (results not shown), rule out the possibility that the multiple bands arise from contaminated primers. Another worker in the

laboratory, using the same batch of *Taq* polymerase, has obtained single bands from different PCR products run on an HA-Y gel. This suggests that the multiple bands are not an artefact of the activity of the *Taq* polymerase.

Whilst the primers were purified by PAGE, this is not the most reliable method, and it is still possible that primers of different lengths entered the final primer preparation, and are responsible for the multiple bands. It may, therefore, be possible to obtain single bands by using non-degenerate primers which have been more rigorously purified, such as by high performance liquid chromatography (HPLC). In using non-degenerate primers, however, it would be necessary to screen the laboratory collection of cyanophages, to determine whether the specificity of the primers has been altered.

It may be preferable to attempt product separation by DGGE using the non-degenerate primers, CPS4 and CPS5, adding a GC-clamp to one, since it is expected that primer degeneracy was the reason for failure of initial attempts (Wilson, personal communication). Indeed, recent results in our laboratory have demonstrated DGGE-separation of cyanophage PCR products using HPLC-purified CPS4 and CPS5 with a GC-clamp (Wilson, personal communication).

Sequence data, obtained from several of the cyanophages in the laboratory, provided complete characterisation of the PCR products. Hence, sequence analysis would be suitable for the characterisation of marine cyanophage populations. However, the large amount of time and labour necessary would make this method less desirable for analysing marine samples. The results (Fig. 5.16) reveal that the amplification region is highly conserved amongst all the cyanophages from which data were obtained, and markedly different from the T4 data, at both the DNA and amino acid levels. Many bases and residues, however, are conserved throughout all the cyanophage sequences, including T4, whilst greater variation is focused on four residues (residue numbers 5, 16, 27 and 37).

The dendrogram resulting from the sequence data (Fig. 5.17) illustrates that the cyanophage sequences are all considerably more different from T4 than from each other. It also shows that there does appear to be some grouping, *i.e.* particular similarity, between cyanophages which were isolated from the same location. Cyanophage strains S-RSM1 and S-RSM2, both isolated from the Red Sea, cluster together, as do cyanophage strains S-MM3, S-MM4 and S-MM5, all isolated from Miami. However, cyanophage strain S-MM1, also isolated from Miami, clusters further away, and is more similar to cyanophage strain S-BnM1, from Bergen, Norway. Hence, whilst cyanophages from one location may be more similar to each other than to cyanophages from a different location, this is not necessarily the case. Similar observations were made with eight viruses of *Micromonas pusilla*, isolated from five different locations, on constructing a dendrogram from DNA hybridisation data (Cottrell and Suttle, 1995); and then with DNA polymerase sequence data from thirteen virus clones, using algal-virus-specific PCR primers (Chen and Suttle, 1996).

Such similarities may reflect the host strains which these cyanophages naturally infect in the environment, perhaps with similar cyanophages infecting the same strain. Cyanophages from the same location which cluster quite separately may therefore infect different strains, given that there may well be a mixture of different host strains in a particular location. On comparison of the dendrogram in Figure 5.17 with Table 5.1, there appears insufficient data to determine whether there would be any correlation between sequence data of the PCR product and host strain specificity. Given the potentially vast range of host strains present in the marine environment (*e.g.* Fuhrman *et al.*, 1993a; Palenik, 1994), one would have to isolate specific cyanophage-host systems in order to determine such a correlation. It is not known whether cyanophages from the same location, yet separated temporally, would show as great similarities as those isolated at the same time. However, due to ocean currents, such as the Gulf Stream, it is probable that this would not be the case.

Chapter 6:

Application of PCR to the Marine Environment

6.1 Introduction

Chapters 4 and 5 of this study describe the design and development of PCR primers which have been shown to be specific for cyanophages which infect marine *Synechococcus* spp.. These chapters suggest that accurate quantification of marine cyanophage populations by PCR is not yet possible, due to the wide genetic diversity of cyanophages. However, a degree of molecular analysis may be obtained either by RFLP or DNA sequence analysis of the PCR product. This Chapter seeks to apply these techniques of PCR and molecular analysis to marine samples in order to obtain completely novel data of the molecular diversity of natural marine cyanophage populations. Previous molecular analysis of marine viruses has largely been performed on virus-host systems which have already been isolated (Chen and Suttle, 1995a; Chen and Suttle, 1996; Cottrell and Suttle, 1995b, Kellogg *et al.*, 1995; Wilson *et al.*, 1993; Wilson, 1994). Very little direct analysis has been made of natural marine virus populations (Chen *et al.*, 1996), and, to date, none has ever been reported of marine cyanophage populations. Marine samples were collected during a scientific cruise from the Falkland Islands to Britain, from which *Synechococcus* spp. were counted, and cyanophage PCR products were amplified and characterised. Additional data were collected during the course of the cruise, by other members of the research team, for comparison with cyanophage data, and are discussed to provide a basis for future work resulting from this study.

The Atlantic Meridional Transect cruise (AMT-2) was the second in a series of transects of the Atlantic Ocean using the Royal Research Ship (RRS) James Clark Ross. The primary objective of this research cruise programme is to investigate biological processes in the open Atlantic Ocean over very broad spacial scales (from 50°S to 50°N for AMT-2). The programme measured physical, chemical, biological and optical variables in the upper 200 m of the water column along a 12000 km transect between Port Stanley (Falkland Islands) and Plymouth (UK).

6.2 Results

6.2.1 Sample collection

Marine seawater samples were collected on board the RRS James Clark Ross on AMT-2 from Port Stanley (Falkland Islands) to Plymouth (UK). The cruise ran from 22nd April (Julian day 113) to 22nd May (Julian day 143) 1996 (1st January is Julian day 1), with a two-day stop-over at Montevideo, Uruguay. The cruise track is displayed in Figure 6.1. Details of each station are recorded in Appendix 5. Figure 6.2 shows the deployment of the sampling rosette bearing collection bottles and CTD (conductivity, temperature, depth) measurement apparatus.

6.2.2 *Synechococcus* spp. counts

Synechococcus spp. counts were made at each station (2.9), at a range of depths above, through and below the thermocline, using water which had been collected for cyanophage concentration (2.11). The data are displayed as a contour plot (Fig. 6.3), illustrating *Synechococcus* spp. abundance throughout the Atlantic transect.

Comparison with Figure 6.4 reveals that peaks in *Synechococcus* spp. abundance occur in the more productive waters near the Falkland Islands, the west coast of Africa, and south-west of Ireland; while the main trough occurs in the unproductive open ocean east of Brazil. The majority of the transect surface waters contained *Synechococcus* spp. concentrations greater than 10^3 ml⁻¹, which is the threshold, observed by Suttle and Chan (1994), necessary for greater cyanophage production (up to 10^5 PFU ml⁻¹). Hence, there should be sufficiently high cyanophage concentrations for detection by PCR in many of the samples (5.3.3).

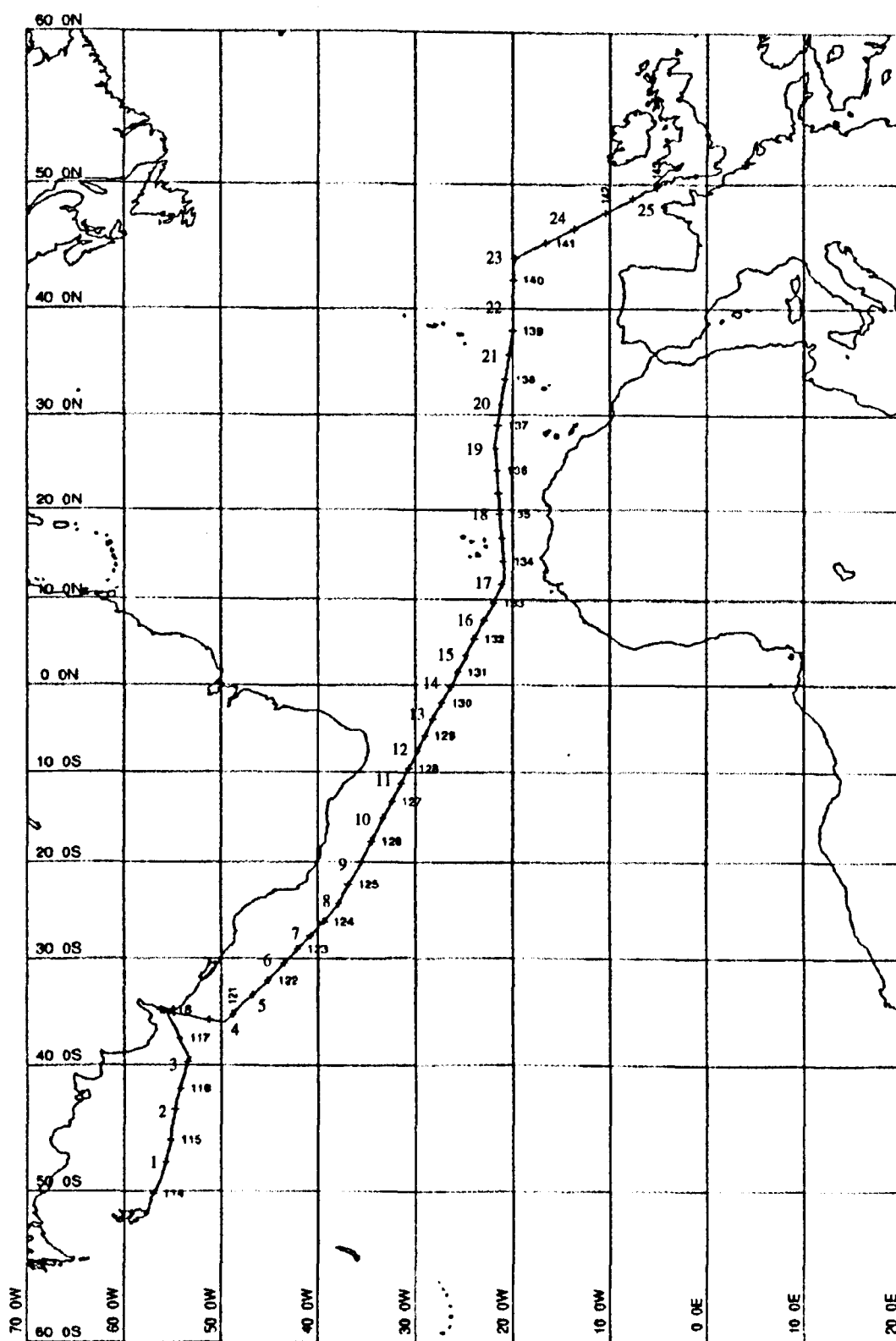


Figure 6.1 AMT-2 cruise track. The cruise ran from Port Stanley (Falkland Islands) to Plymouth (UK), on board the RRS James Clark Ross. Degrees latitude and longitude are shown. Station numbers are marked to the left of the track; the Julian day is marked to the right. The map is a Mercator projection on a scale of 1:55661934 at latitude -0, reproduced from the AMT-2 cruise report by David Robins.

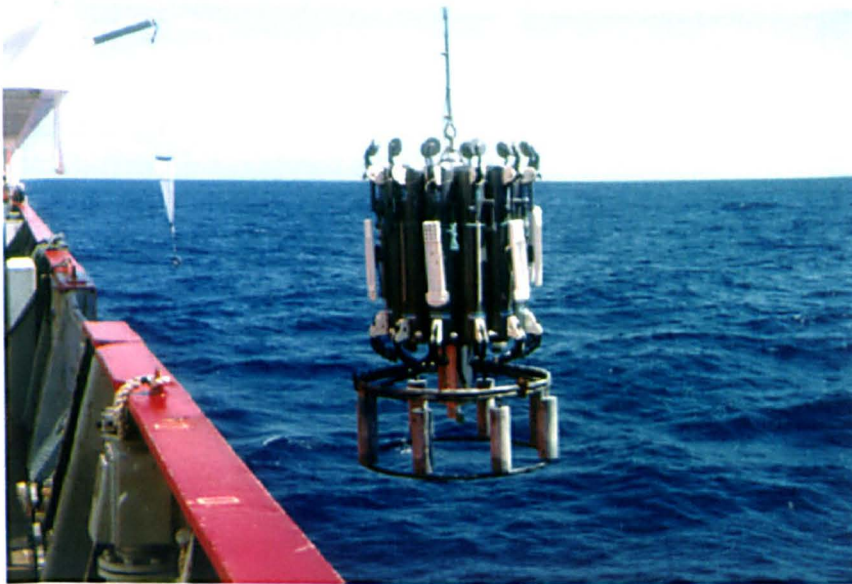


Figure 6.2 Sampling apparatus being deployed mid-cruise. The sampling rosette contains collection bottles and CTD apparatus, for the collection of *Synechococcus* spp. and cyanophages.

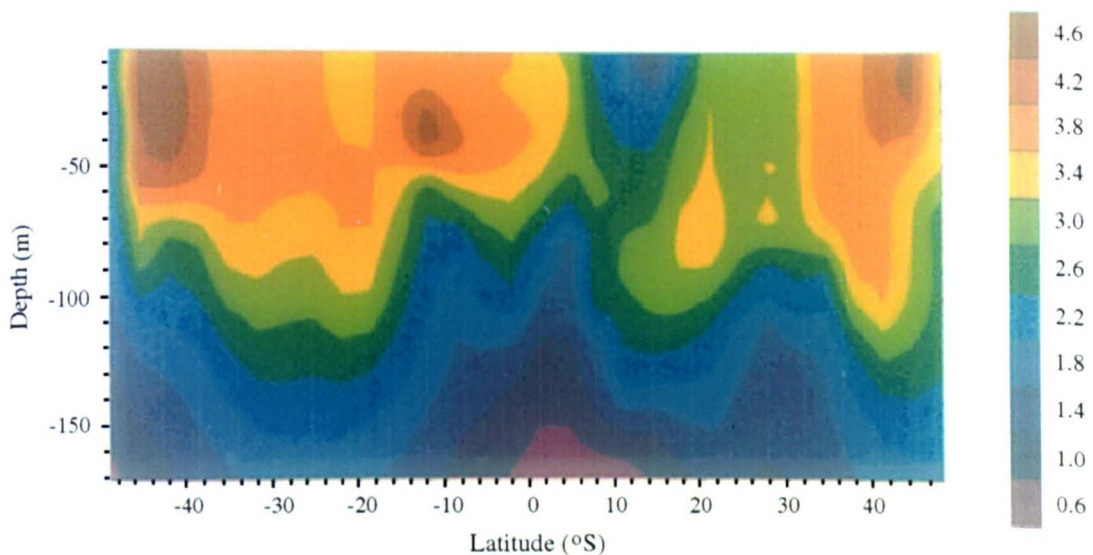


Figure 6.3 Contour plot of *Synechococcus* spp. abundance on AMT-2. *Synechococcus* spp. counts were made by epifluorescence microscopy, and are plotted as the \log_{10} (cells ml^{-1}). Data were collected on board the RRS James Clark Ross, between Port Stanley (Falkland Islands) and Plymouth (UK) in the boreal spring, 1996. Latitude is in degrees south.

6.2.3 AMT-2 cruise data

A comparison was made of the *Synechococcus* spp. data (Fig. 6.3) with composite

data of chlorophyll *a* concentration, averaged from several years (Fig. 6.4), to give a general idea of the context of the AMT-2 data (see above). Comparisons were also made of the *Synechococcus* spp. data with other data collected on AMT-2 (Fig. 6.5).

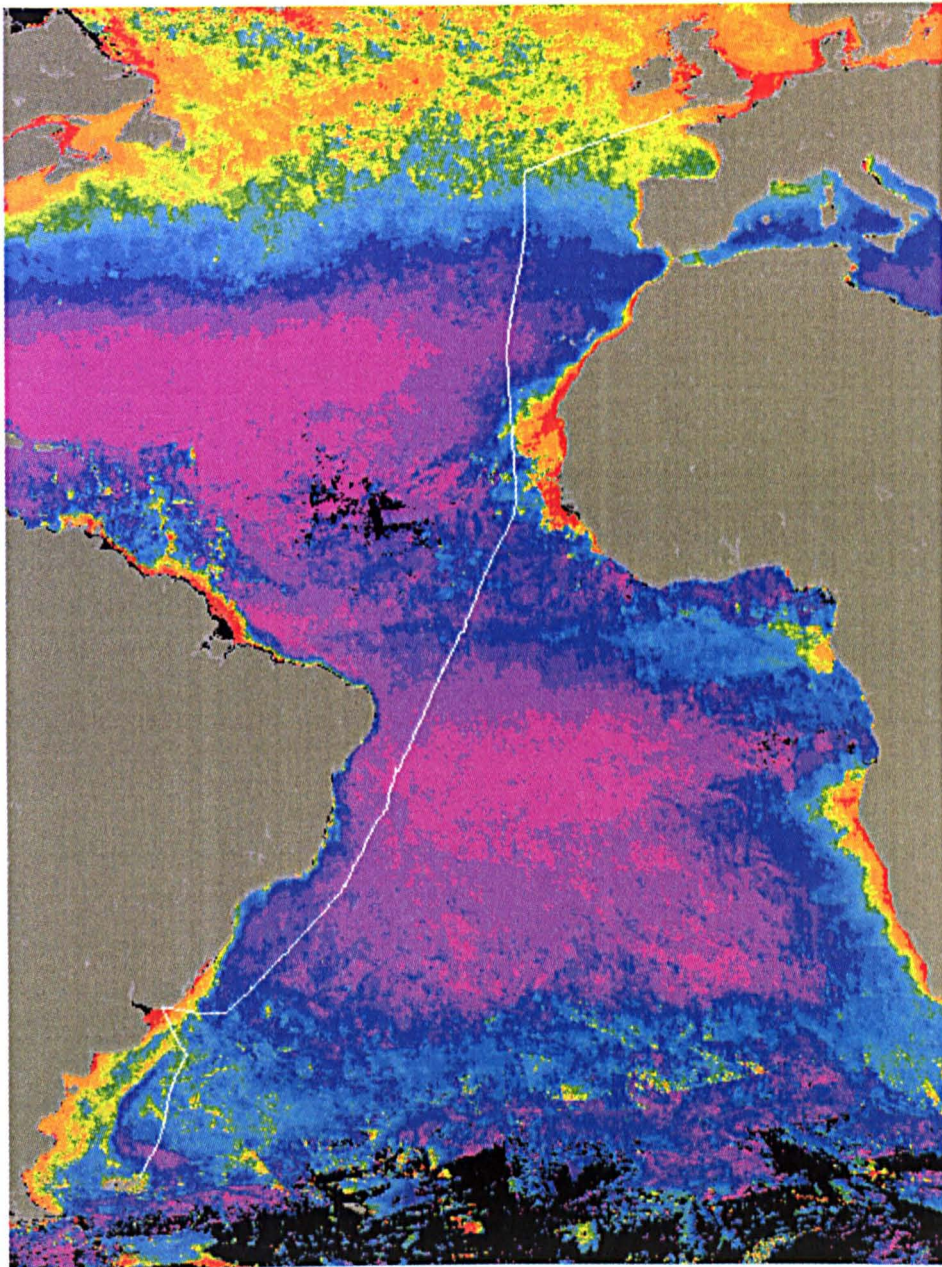


Figure 6.4 Composite satellite data of chlorophyll *a* concentrations in the Atlantic Ocean. Data are averaged over several years, for the period of the AMT-2 cruise (boreal spring). Greens, yellows and reds represent high concentrations; blues and purples represent low concentrations. The AMT-2 cruise track, from Port Stanley to Plymouth, is marked in white. Reproduced from the AMT-2 cruise report by David Robins.

(a) Temperature ($^{\circ}\text{C}$)

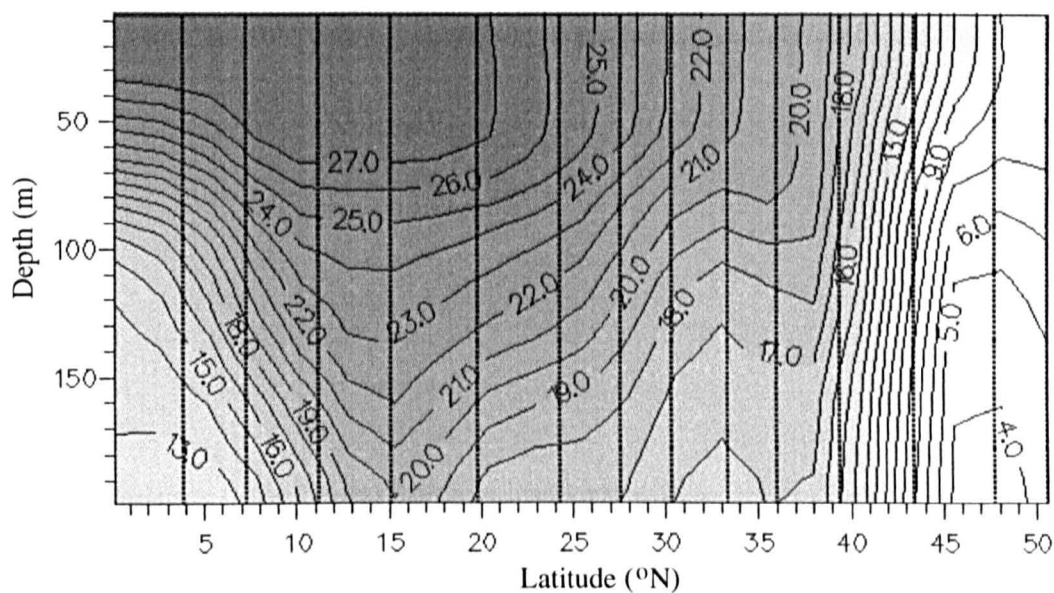
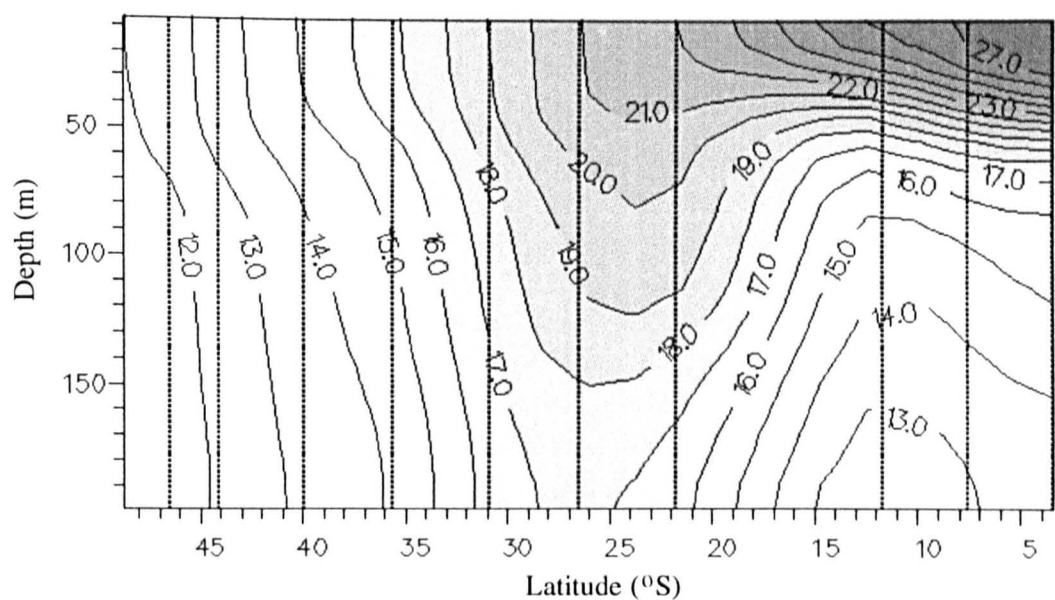
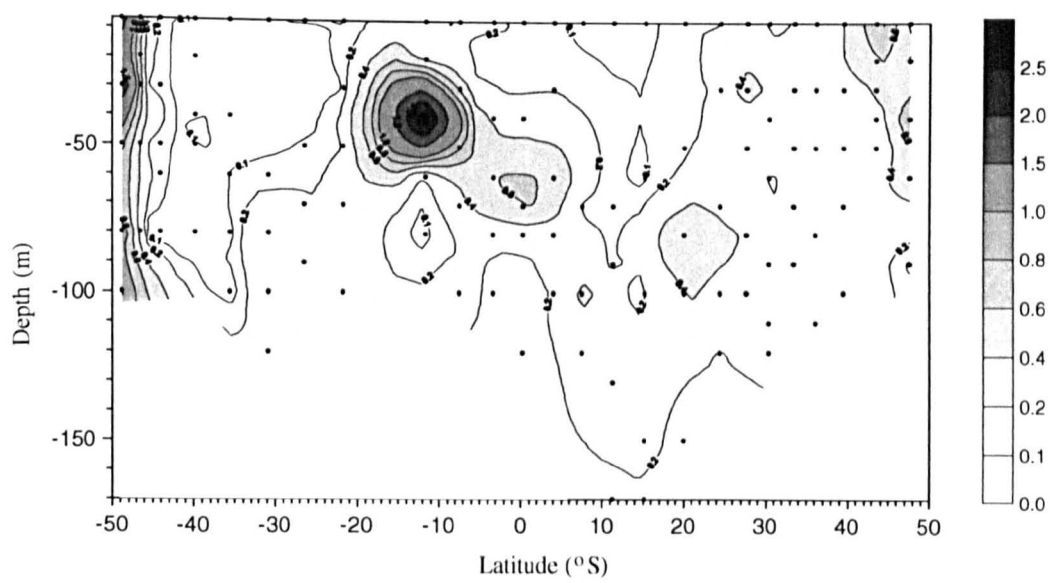
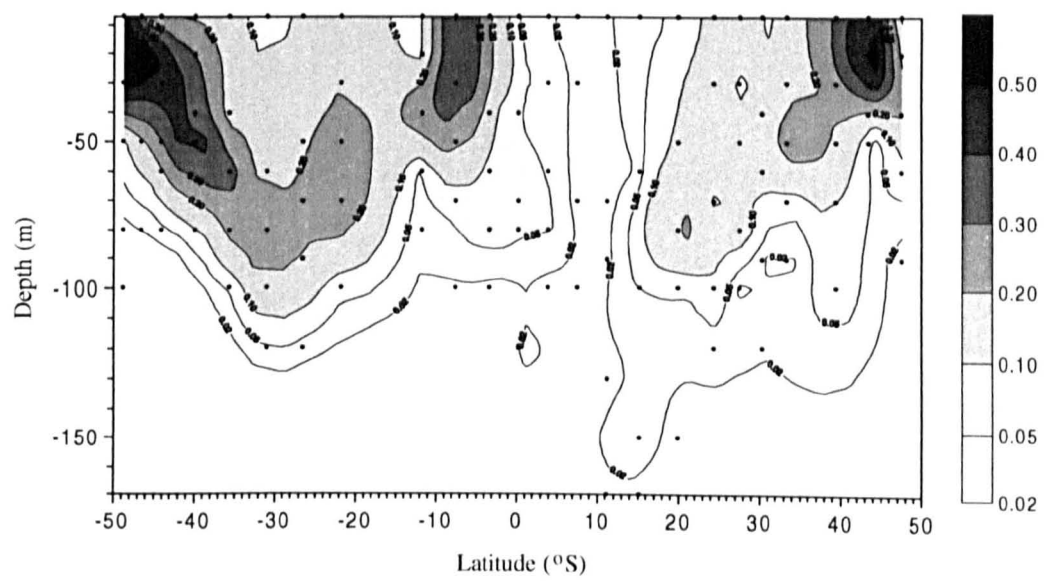


Figure 6.5 AMT-2 cruise data. Data were collected on board the RRS James Clark Ross, between Port Stanley (Falkland Islands) and Plymouth (UK) in the boreal spring, 1996, by members of the AMT-2 team. Latitude is in degrees south. Contour plots a-d were reproduced from the AMT-2 cruise report by David Robins. Equatorial data for temperature (a) are absent, and the north-south axis for temperature is reversed relative to plots b-f. Contour plots e and f were obtained from Alan Pomroy. Continued below.

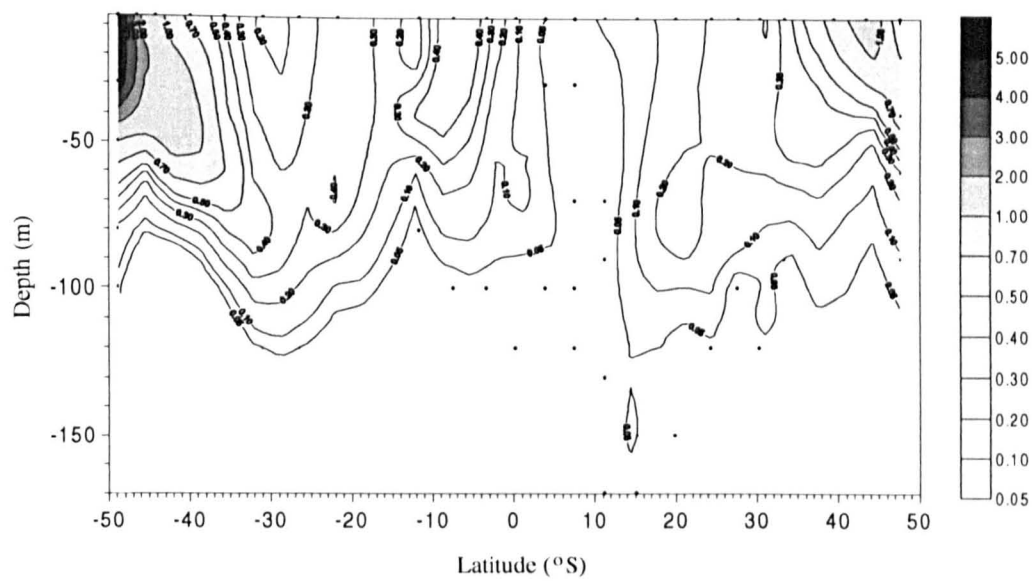
(b) Chlorophyll *a* concentration (mg m⁻³)



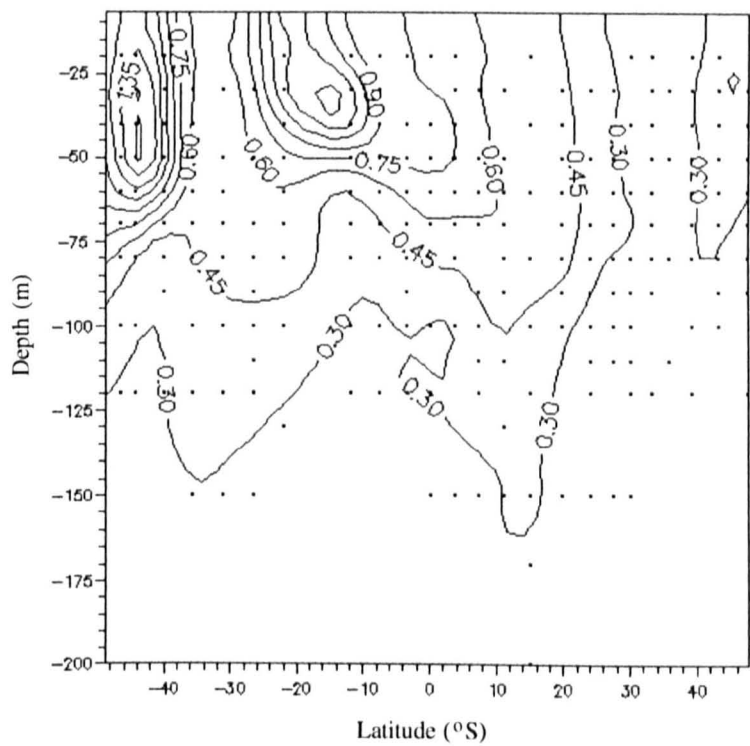
(c) Photosynthesis by the 0.2-2 μm fraction (mgC m⁻³ h⁻¹)



(d) Total photosynthesis ($\text{mgC m}^{-3} \text{ h}^{-1}$)



(e) Bacteria ($\times 10^6 \text{ ml}^{-1}$)



(f) Nitrate concentration (mg m^{-3})

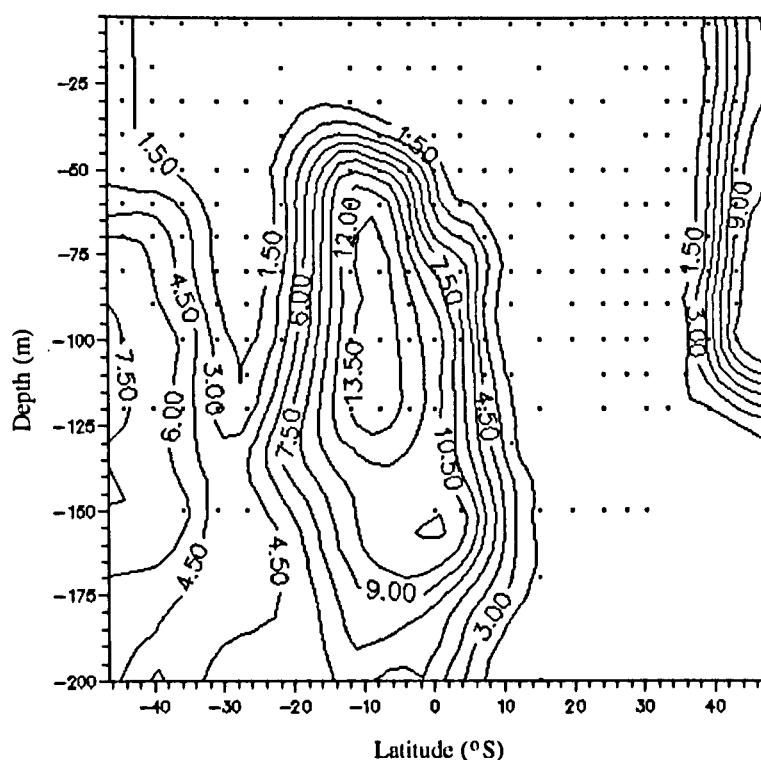


Figure 6.5 (continued) AMT-2 cruise data. Data were collected on board the RRS James Clark Ross, between Port Stanley (Falkland Islands) and Plymouth (UK) in the boreal spring, 1996, by members of the AMT-2 team. Latitude is in degrees south. Contour plots a-d were reproduced from the AMT-2 cruise report by David Robins. Contour plots e and f were obtained from Alan Pomroy.

6.2.4 PCR of cruise samples

Results of experiments to determine the sensitivity of the cyanophage-specific PCR primers, CPS1 and CPS2 (5.3.3), suggested that it may be advisable to concentrate cyanophages from seawater samples before attempting PCR amplification. Hence, samples were concentrated *ca.* 100-fold on board the research ship by tangential flow filtration (2.11.1) (Append. 5). The AMT-2 *Synechococcus* spp. count (Fig. 6.3) suggested that there should be sufficient cyanophages for PCR detection in the majority of 7m samples along the transect; hence, no further concentration was made. PCR was then performed on 1 μl of concentrate from the 7 m sample from each

station (2.8.10.1) (Fig. 6.6). A smaller product appears to be present in some samples. This may well result from primer dimerisation, as the bands are a similar size to primer dimers observed in other reactions (not shown). The location of each station is shown in Figure 6.1 and, with grid references, in Appendix 5.

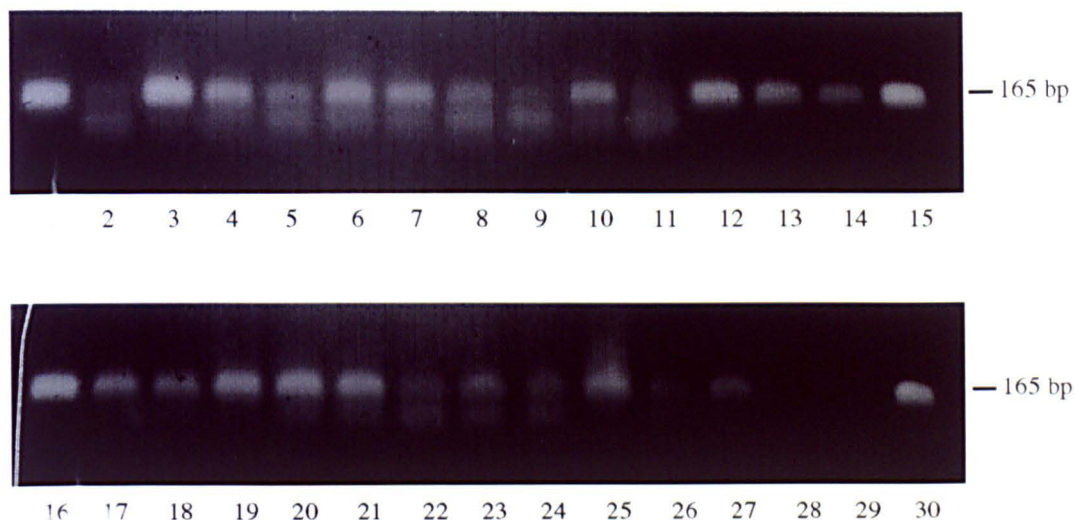


Figure 6.6 PCR amplification of cruise samples using cyanophage-specific primers, CPS1 and CPS2. Samples were collected on board the RRS James Clark Ross, between Port Stanley (Falkland Islands) and Plymouth (UK) in the boreal spring, 1996, and concentrated 100-fold by tangential flow filtration. PCR was performed on 1 μ l of concentrate from the 7 m sample from each station. Station 1 is nearest Port Stanley. The resulting reaction mixtures, most containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h. Tracks 1, 15, 16 and 30 contain products from cyanophage strain S-BnM1 as positive controls; track 29 contains no target DNA as a negative control; tracks 2-14 contain products from stations 1-13; tracks 17-28 contain products from stations 14-25.

An attempt was made to characterise PCR products which had been amplified from cruise samples, by RFLP analysis, using the restriction endonuclease *Hinf*I. This enzyme was found to give three distinct restriction patterns amongst cultured cyanophages (Fig. 5.13). The results revealed the presence of all three restriction patterns for each of the cruise products; thus, each station produced an identical restriction pattern (Fig. 6.7).

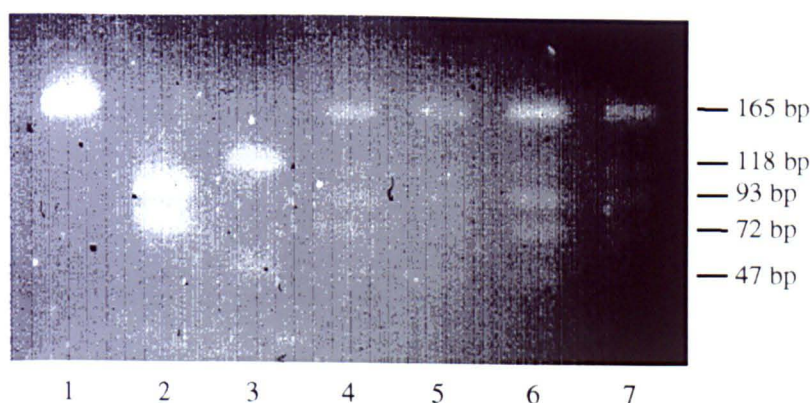


Figure 6.7 RFLP analysis of PCR products from AMT-2. PCR products were amplified using cyanophage-specific primers, CPS1 and CPS2, from seawater collected between Port Stanley (Falkland Islands) and Plymouth (UK). Products were restricted with *HinfI* and run on a 3% (w/v) agarose gel at 50 mA for 3 h. Tracks 1-3 contain restricted products from cyanophage strains (1) S-BnM1; (2) S-WHM1; and (3) S-PM2. Tracks 4-7 contain restricted products from cruise stations (4) 3; (5) 4; (6) 5; and (7) 16.

PCR products from the cruise samples were characterised by sequence analysis. Products had been obtained from samples taken from three depth profiles (results not shown). However, due to limited time and resources, PCR products from only the surface (7 m) samples from six stations were analysed. Sequence data were obtained from stations which resulted in relatively large amounts of PCR product, but which were widely separated geographically (stations 2, 6, 11, 16, 18 and 23). These products were subsequently ligated into the TA vector, pCR2.1, and several resulting transformants from each station were sequenced on both strands (2.8.11). Each transformant potentially contained a different cyanophage sequence. A few different sequences were obtained from four of the stations, but work was concentrated upon obtaining many sequences just from stations 2 and 18. A GCG "Pileup" alignment was made of the DNA and amino acid sequences of the cruise products along with the products of the cultured cyanophages and T4 (Fig. 6.8).

The alignments reveal the high degree of conservation of this sequence amongst marine cyanophages, and some identical sequences within a station (*e.g.* 2/1, 2/5, 2/6, 2/8, 2/14, 2/16, 2/23 and 2/39). The frameshift apparent in S18/1 of Figure 6.8b may be an artefact of the PCR. The DNA alignment, with identical cyanophage sequences

(a)

```
S-PM2      : TAATCTACCAAAAGCTAAAGGCAGAACCAATACTTGCCTGATGTAATGT : 47
S-WHM1     : TAATCTCCCCAAGAACAAGGCGAACCAATATCTCCGTGAAGTTATGA : 47
S-BnM1     : CAATCTTCCAAAGGTAAAAGCAGAACCAATACCTCAGAGAGGTAATGG : 47
S-RSM1     : TAATCTGCCCAAAATGAAGGCAGAACCAATATCTGCCGATGTTATGA : 47
S-RSM2     : TAATCTGCCCAAAATGAAGGCAGAACCAATATCTGCCGTGATGTAATGA : 47
S-MM1      : CAATCTTCCGAAGGTAAAGGCAGAACCAATACCTTCGTGAAATCATGA : 47
S-MM3      : TAATCTACCTAAGGTCAAAGCAGAACCAATACTTGCCTGATGTCATGT : 47
S-MM4      : CAATCTACCTAAGGTCAAAGCAGAACCAATACTTGCCTGATGTCATGA : 47
S-MM5      : CAATCTACCTAAAAGTCAAAGCGAACCAATATCTCCGTGATGTAATGG : 47
S2/1       : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/5       : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/6       : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/8       : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/9       : TAACTTGCCGAAAGTAAAAGCAGAACGCTACCTCAAAGATGTGATGA : 47
S2/14      : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/16      : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/23      : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/25      : TAACTTGCCCTAAGGTTAAGGCAGAACCAATATCTGCCGTGACGTTATGC : 47
S2/34      : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/36      : CAATCTTCCCTAAACAAAAGGCAGAGCAGTATCTGCCGTGATGTGATGA : 47
S2/38      : AAATCTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S2/39      : CAATCTTCCCTAAAGCAAAGGCAGAGCAATACCTCAGAGAGGTTATGT : 47
S6/7       : CAATCTACCCAAGATAAAAGCAGAGCAATATCTTAAAGATGTGATGA : 47
S6/13      : TAATCTACCAAAAAGTAAAAGCAGAACCAATACCTCAAAGATGTGATGA : 47
S6/21      : TAATCTTCCAAAAGTAAAATCTGAGCAATACCTCAGAGATGTTATGA : 47
S11/4      : TAACTTGCCCTAAAGTAAAGGCAGAGCAATACCTTCGTGATGTTATGG : 47
S11/22     : AAATCTGCCGAAGATAAAAGCAGAGCAATATCTCAGAGATGTGATGA : 47
S16/2      : TAATCTTCCAAAAGTAAAATCTGAGCAATACCTCAGAGATGTCATGA : 47
S16/10     : TAATCTACCAAAAATTAAAGCAGAACCAATATCTGAAAGATGTTATGA : 47
S16/13     : TAATCTTCCAAAAGTAAAATCTGAGCAATACCTCAGAGATGTCATGA : 47
S18/1      : TAATCTACCTAAGGTAAAGGCAGAACCAATATCTGCCGTGATGTCATGT : 47
S18/3      : CAATCTGCCAAAAGTAAAGGCAGAACCAATATCTTCGTGATGTGATGA : 47
S18/7      : TAATCTACCAAAAGGTAAAGGCAGAACCAATACCTGCCGTGATGTAATGA : 47
S18/9      : CAATCTACCTAAAAGTCAAAGCGAACCAATATCTCCGTGACGTAATGG : 47
S18/15     : CAATCTGCCAAAAGTAAAGGCAGAACCAATATCTTCGTGATGTGATGA : 47
S18/17     : CAATCTGCCAAAAGTAAAGGCAGAACCAATATCTTCGTGATGTGATGA : 47
S18/18     : CAATCTGCCAAAAGCAAAGGCAGAACCAATATCTTCGTGATGTGATGA : 47
S18/34     : CAATCTGCCAAAAGTAAAGGCAGAACAGTATCTTCGTGATGTGATGA : 47
S18/35     : CAATCTGCCAAAAGTAAAGGCAGAACCAATATCTTCGTGATGTGATGA : 47
S18/36     : CAATCTGCCCTAAAGTTAAGGCAGAACCAATATCTGCCGTGATGTTATGC : 47
S18/37     : TAACTTGCCGAAAGTAAAGGCAGAACGCTACCTCAAAGATGTGATGA : 47
S18/41     : TAATCTACCAAAAATTAAAGCAGAACCAATATCTGAAAGATGTTATGA : 47
S23/5      : TAATCTACCTAAAAGTCAAAGCAGAGCAGTATCTACAGCAGGTAATGA : 47
S23/10     : TAATCTACCTAAAAGTCAAAGCAGAGCAGTATCTACAGCAGGTAATGA : 47
T4         : TAATATGCCTGCTCGTAAAGCTCTGAGCACATGCAACATGTTATGA : 47
```

S-PM2	:	CACGTTACAGAAACAAGCTTGGTTACGATGGTCAAAACAGGAGAGATT	:	94
S-WHM1	:	GTCCGTTATAGAAACAAGATGGTCTATGATGCAAACACTGCCGAGATT	:	94
S-BnM1	:	GACGTTACCGCAACAAACTTGGTTATGATGCAAACAGAGCTGAAATC	:	94
S-RSM1	:	TGACATATCGTAACAAGCTTGTGTATGATGCAAACACTGCAGAGATT	:	94
S-RSM2	:	TGCGTTATCGCAACAAACTTGTATACGATGCAAACAGAGAGAGATC	:	94
S-MM1	:	ATCGTTATCGTTCTAAACTTGTATATGATGCTAACAGGCGTGAGGTT	:	94
S-MM3	:	CTCGCTATCGTAATAAGCTAGTCTATGATGCACAGACTGCTGAGATG	:	94
S-MM4	:	GTCCGTTATCGCAACAAGCTAGTGTATGACGCAAACACTGCTGAAATG	:	94
S-MM5	:	CACGTTATCGTAACAACACTAGTTTACGATGCCAGTACTGCAGAGATC	:	94
S2/1	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/5	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/6	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/8	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/9	:	ATCGTTATCGTAACAAGTTGGTGTATGATGCACGAACTGCTGAGATT	:	94
S2/14	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/16	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/23	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/25	:	AGACATATCGTAACAACACTTGTGTATGTTTCTAGCACTGCTGAAATT	:	94
S2/34	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/36	:	TGCGTTATAGAAACAAGTTAGTCTATGATGCAAACAGGCGTGAACCT	:	94
S2/38	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S2/39	:	CTCGCTACAGAAACAAGTTAGTGTATGACGCTAACACTGCTGAAATT	:	94
S6/7	:	ATCCGTTATCGTAACAAGTTGGTGTATGATGCATCAACTGCTGAAATA	:	94
S6/13	:	ATCCGTTATCGTAACAAGTTGGTGTATGATGCATCCTACTGCTGAGATT	:	94
S6/21	:	ATCGTTATCGTAACAACACTAGTTTACGATGCAAACACTGCTGAGATT	:	94
S11/4	:	CTCGCTATCGTAACAACACTAGTTTACGATGCTGCTACTGCTGAAATT	:	94
S11/22	:	TGCGATATCGAAACAACACTAGTTTACGACGCTAACACAGGAGAGATC	:	94
S16/2	:	ATCGTTATCGTAACAACACTAGTTTACGATGCAAACACTGCTGAGATT	:	94
S16/10	:	ATCCGTTATCGTAACAACACTGGTATATGATGCATCTACTGCTGAAATC	:	94
S16/13	:	ATCCGTTATCGTAACAACACTAGTTTACGATGCAAACACTGCTGAGATT	:	94
S18/1	:	CTCGCTATCGT.ATAAGTTGGTTTACAATGCCAACACTGCTGAAATT	:	93
S18/3	:	TGCGTTATCGCAACAAACTTGTATATGATGCAAACAGAGAGAGATT	:	94
S18/7	:	GTCCGTTACAGAAACAACACTTGTATACGATGCAAACACTGCAGAGATC	:	94
S18/9	:	CACGTTACCGTAACAACACTTGGTTTACGATGCTGCTACTGCAGAGATC	:	94
S18/15	:	TGCGTTATCGCAACAAACTTGTATATGATGCAAACAGAGAGAGATT	:	94
S18/17	:	TGCGTTATCGCAACAAACTTGAATATGATGCAAACAGAGAGAGATT	:	94
S18/18	:	TGCGTTATCGCAACAAACTTGTATATGATGCAAACAGAGAGAGATT	:	94
S18/34	:	TGCGTTATCGCAACAAACTTGTATATGATGCAAACAGAGAGAGATT	:	94
S18/35	:	TGCGTTATCGCAACAAACTTGTATATGATGCAAACAGAGAGAGATT	:	94
S18/36	:	AGACATATCGTAACAACACTTGTGTATGATGCACAGACTGCTAAAGTT	:	94
S18/37	:	ATCGTTATCGAAACAAGTTGGTGTATGATGCACGAACTGCTGAGATT	:	94
S18/41	:	ATACATATCGTAACAACACTAGTGTATGATGCATCTGTGGTGAATC	:	94
S23/5	:	ATCGTTATAGGAACAAGTTATCCATATAATGCTCAGACTGGTGAGATG	:	94
S23/10	:	ATCGTTATAGGAACAAGTTAACTTATAATGCTCAGACTGCAGAGATG	:	94
T4	:	ACACGATGAAAACCGTGTAGTATATGATGCATCAACAGGTAAATA	:	94

S-PM2	:	CGTGATGATAAAAAAGCATATGCTCT	:	118
S-WHM1	:	AAAGACGACAAGAAATTTATGTCA	:	118
S-BnM1	:	AAGGACGACAAGAAACATATGTCG	:	118
S-RSM1	:	CGTGATGATAAAAAATACATGGCA	:	118
S-RSM2	:	CGTGATGACAAAAAGTACATGGCG	:	118
S-MM1	:	AGAGACGATAAGAAGTTCATGCTCT	:	118
S-MM3	:	CGTGATGACAAAAAGCACATGAGT	:	118
S-MM4	:	CGTGATGACAAAAAGCATATGAGT	:	118
S-MM5	:	CGTGATGACAAAAAGCATATGAGT	:	118
S2/1	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/5	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/6	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/8	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/9	:	CGTGATGATAGAAATCATATGCTCT	:	118
S2/14	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/16	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/23	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/25	:	CGTGATGATAAAAAAGCATATGAGT	:	118
S2/34	:	CGTGACGGCAAAAAAGTTCATGAGT	:	118
S2/36	:	CGTGATGATAAGAAGTTCATGCTCT	:	118
S2/38	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S2/39	:	CGTGACGACAAAAAGTTCATGAGT	:	118
S6/7	:	CGAGATGATCGTAATCAAATGAGT	:	118
S6/13	:	AGGGACGATAGAAATCATATGAGT	:	118
S6/21	:	CGTGATGATCGTAAGCACATGAGT	:	118
S11/4	:	CGTGACGACAAAAAGCATATGAGT	:	118
S11/22	:	CGCGATGACAAGAAGTACATGGCA	:	118
S16/2	:	CGTGATGACCGCAAGCACATGAGT	:	118
S16/10	:	AGAGATGACCGAAACCATATGTCA	:	118
S16/13	:	CGTGATGACCGCAAGCACATGAGT	:	118
S18/1	:	CGTGACGACAAAAAGATGATGTCC	:	117
S18/3	:	CGTGATGACAAAAAGTACATGGCG	:	118
S18/7	:	AAGGATGACAAGAAATTCATGCTCT	:	118
S18/9	:	CGTGATGACAAAAAGCATGTGAGT	:	118
S18/15	:	CGTGATGACAAAAAGTACATGGCG	:	118
S18/17	:	CGTGATGACAAAAAGTACATGGCG	:	118
S18/18	:	CGTGATGACAAAAAGTACATGGCG	:	118
S18/34	:	CGTGATGACAAAAAGTACATGGCG	:	118
S18/35	:	CGTGATGACAAAAAGTACATGGCG	:	118
S18/36	:	CGTGACGATAAAAAAGTATATGTCA	:	118
S18/37	:	CGTGACGATAGAAATCATATGCTCT	:	118
S18/41	:	AGAGATGACCGAAACCATATGTCA	:	118
S23/5	:	AAAAACGATAAAAAAGTAATGCTCT	:	118
S23/10	:	AAGAACGACAAAAAGTAATGCTCT	:	118
T4	:	AAAAATCAACAGCATAATATGCTCT	:	118

Figure 6.8 "Pileup" alignment of sequences of PCR products amplified from cruise samples using cyanophage-specific PCR primers (CPS1 and CPS2). Alignments were made, using the software package GCG, of (a) the DNA; and (b) the amino acid sequences of AMT-2 cruise products, together with the cultured cyanophage amplification products and the equivalent region in T4 (5.3.5.3). Sequence data only between the primers are shown. Conserved bases and residues are highlighted to four levels. Cultured cyanophage strains are preceded by "S-". The first number of the cruise products represents the station number. Continued overleaf.

(b)

```
S-PM2      : NLPKVKAEQYL RDVMSRYRNKLVDGQTGEIRDDKKHMS : 39
S-WHM1     : NLPKNKAEQYLREVM SRYRNKMVYDANTGEIKDDKKFMS : 39
S-BnM1     : NLPKVKAEQYLREVMGRYRNKLVDANTGEIKDDKKHMS : 39
S-RSM1     : NLPKMKAQYLRDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S-RSM2     : NLPKMKAQYLRDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S-MM1      : NLPKVKAEQYLREVMNRYR SKLVDANTGEVRDDKKFMS : 39
S-MM3      : NLPKVKAEQYL RDVMSRYRNKLVDANTGEMRDDKKHMS : 39
S-MM4      : NLPKVKAEQYL RDVMSRYRNKLVDANTGEMRDDKKHMS : 39
S-MM5      : NLPKVKAEQYL RDVMARYRNKLVDASTGEIRDDKKHMS : 39
S2/1       : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/5       : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/6       : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/8       : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/9       : NLPKVKAEAYLKDV MNRYRNKLVDARTGEIRDDRNHMS : 39
S2/14      : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/16      : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/23      : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/25      : NLPKVKAEQYL RDVMQRYRNKLVDV SSTGEIRDDKKHMS : 39
S2/34      : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDGKKFMS : 39
S2/36      : NLPKQKAEQYL RDVM MRYRNKLVDANTGELRDDKKFMS : 39
S2/38      : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S2/39      : NLPKAKAEQYLREVM SRYRNKLVDANTGEIRDDKKFMS : 39
S6/7       : NLPKIKAEQYLKDV MNRYRNKLVDASTGEIRDDRNQMS : 39
S6/13      : NLPKVKAEQYLKDV MNRYRNKLVDASTGEIRDDRNHMS : 39
S6/21      : NLPKVKSEQYLRDVM NRYRNKLVDANTGEIRDDRKHMS : 39
S11/4      : NLPKVKAEQYL RDVMARYRNKLVDAA TGEIRDDKKHMS : 39
S11/22     : NLPKIKAEQYL RDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S16/2      : NLPKVKSEQYLRDVM NRYRNKLVDANTGEIRDDRKHMS : 39
S16/10     : NLPKIKAEQYLKDV MNRYRNKLVDASTGEIRDDRNHMS : 39
S16/13     : NLPKVKSEQYLRDVM NRYRNKLVDANTGEIRDDRKHMS : 39
S18/1      : NLPKVKAEQYL RDVMSRYR ISWFTMPTLVKFVTTKR *C. : 37
S18/3      : NLPKVKAEQYL RDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S18/9      : NLPKVKAEQYL RDVMARYRNKLVDAA TGEIRDDKKHVS : 39
S18/15     : NLPKVKAEQYL RDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S18/17     : NLPKVKAEQYL RDVM MRYRNKL EYDANTGEIRDDKKYMA : 39
S18/18     : NLPKAKAEQYL RDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S18/34     : NLPKVKAEQYL RDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S18/35     : NLPKVKAEQYL RDVM MRYRNKLVDANTGEIRDDKKYMA : 39
S18/36     : NLPKVKAEQYL RDVMQRYRNKLVDAA TCKVRDDKKYMS : 39
S18/37     : NLPKVKAEAYLKDV MNRYRNKLVDARTGEIRDDRNHMS : 39
S18/41     : NLPKIKAEQYLKDV MNRYRNKLVDASA GEIRDDRNHMS : 39
S23/5      : NLPKVKAEQYLQQVM NRYRNKL SYNAQTGEMKNDKKVMS : 39
S23/10     : NLPKVKAEQYLQQVM NRYRNKL TYNAQTGEMKNDKKVMS : 39
T4         : NMPARKAAEHMQHVMNTMKNRVYDASTGKIKXNQHNMS : 39
```

Figure 6.8 (continued) "Pileup" alignment of sequences of PCR products amplified from cruise samples using cyanophage-specific PCR primers (CPS1 and CPS2). Alignments were made, using the software package GCG, of (a) the DNA; and (b) the amino acid sequences of AMT-2 cruise products, together with the cultured cyanophage amplification products and the equivalent region in T4 (5.3.5.3). Sequence data only between the primers are shown. Conserved bases and residues are highlighted to four levels. Cultured cyanophage strains are preceded by "S-". The first number of the cruise products represents the station number.

and T4 removed, was then analysed using DNA DISTANCE and NEIGHBOUR analyses from PHYLIP version 3.5c (Felsenstein, 1993), and an unrooted dendrogram was consequently constructed (Fig. 6.9), illustrating the degree of similarity between the different sequences. Figure 6.9 illustrates that some sequences from the same station cluster closely together (*e.g.* 23/5 and 23/10), whereas some are more similar to other sequences from different locations than to those from their own station (*e.g.* 2/9 and 18/37).

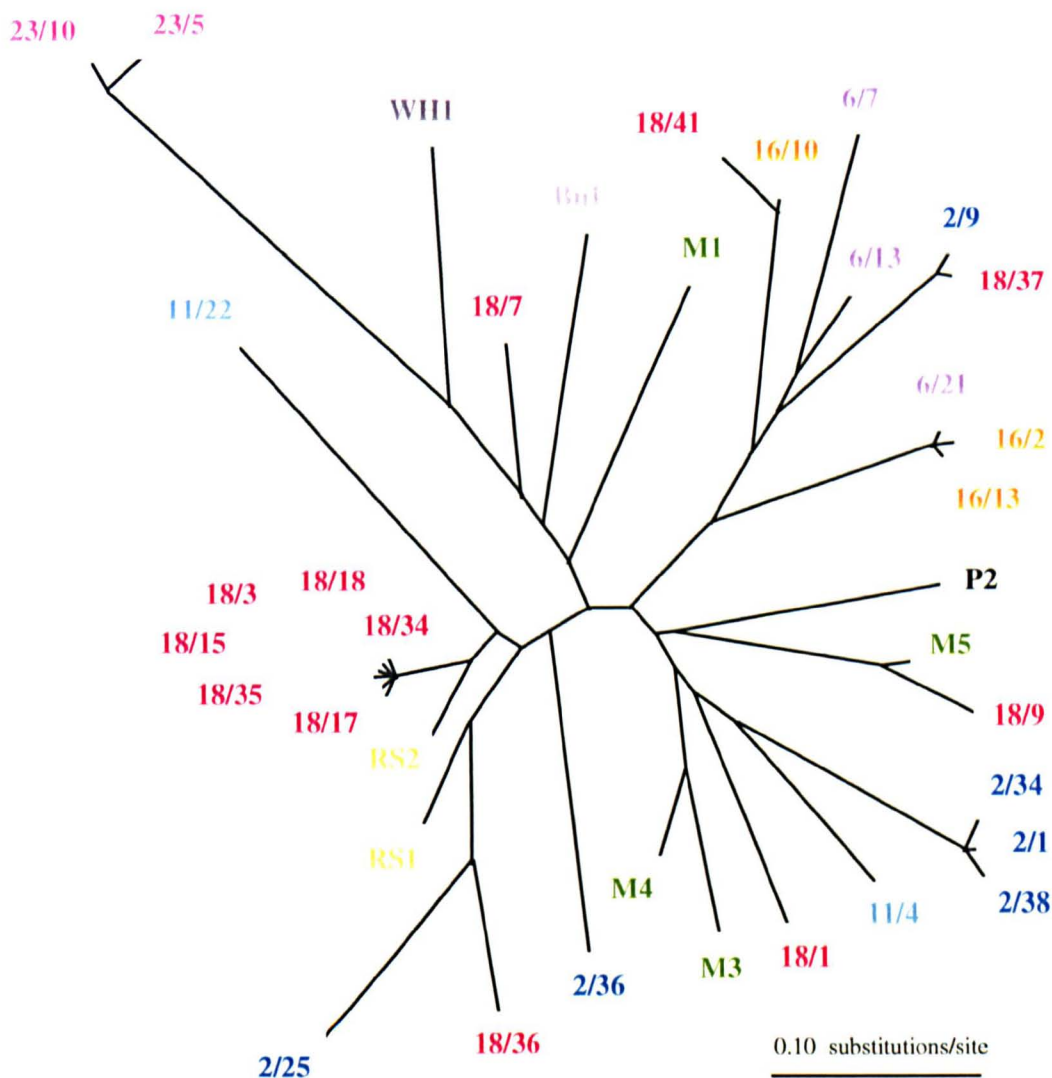


Figure 6.9 Unrooted dendrogram of PCR products amplified from cruise samples using the cyanophage-specific primers, CPS1 and CPS2. The dendrogram was constructed using PHYLIP version 3.5c (Felsenstein, 1993) analysis of a GCG "Pileup" alignment of the DNA sequences of AMT-2 cruise products, together with the cultured cyanophage amplification products (5.3.5.3). Only the sequence data between the primers were used. Cultured cyanophage strains are abbreviated (*e.g.* Bn1 instead of S-BnM1), and the first number of the cruise products represents the station number.

6.3 Discussion

6.3.1 PCR of cruise samples

6.3.1.1 Detection of marine cyanophages

Tangential flow filtration has been successfully used to concentrate marine picoplankton (Giovannoni *et al.*, 1990b). Concentration of cyanophages by tangential flow filtration proved to be a cheap, practical, technically simple and reasonably effective method for use on board the ship. Any quantitative assay of cyanophages from marine samples would require the concentration efficiency to be calculated, which may decrease with time (see below). Other methods which have been used to efficiently concentrate marine viruses include vortex flow filtration (Paul *et al.*, 1991) and ultrafiltration (Suttle *et al.*, 1991a).

The result of the attempted PCR on the cruise samples (Fig. 6.6) shows that PCR products were obtained from many of the samples, even though they had been stored for 11 months at -20°C before PCR analysis. These results clearly show the usefulness and sensitivity of PCR in detecting marine cyanophages in the natural environment. They suggest that cyanophages, in particular, cyanomyoviruses which infect *Synechococcus* spp., are present throughout the Atlantic Ocean, in both hemispheres. Marine cyanophages have been observed in many oceanographic locations (Wilson, 1994), and a few analyses have been made along transects (*e.g.* Suttle and Chan, 1993; 1994). Such studies show the ubiquitous nature of marine cyanophages; so their presence throughout the Atlantic Ocean is not unexpected.

Suttle and Chan (1993; 1994) observed sufficiently high concentrations of infectious cyanophages to be detected by PCR ($>10^3$ ml⁻¹) throughout transects in the Gulf of Mexico. Seasonal studies have shown that cyanophage concentrations in winter may go below the limit of detection by PCR (Waterbury and Valois, 1993); however, these corresponded to *Synechococcus* spp. concentrations which were lower than

those generally found throughout this study. Hence, it is not unexpected that cyanophages should be found in sufficiently high concentrations for PCR detection throughout most of the transect. Indeed, comparison of the *Synechococcus* spp. counts (Fig. 6.3) with the threshold for cyanophage multiplication observed by Suttle and Chan (1994) suggests that infective cyanophage concentrations should be *ca.* 10⁵ ml⁻¹ for much of the transect.

PCR products were faint towards the end of the cruise. This is probably a result of the filter unit clogging up, as samples were noticed to take increasingly longer times to be concentrated towards the end of the cruise. Hence, the low product intensity from stations 19-25 may not necessarily reflect *in situ* cyanophage concentrations. Even in the earlier stages of the cruise the amount of PCR product does not necessarily reflect the number of cyanophages present, since faint products were obtained from samples which contained relatively high *Synechococcus* spp. concentrations (*e.g.* station 4; Fig. 6.3), and should therefore have contained high cyanophage concentrations (Suttle and Chan, 1994). This observation coincides with previous observations of very faint PCR products even from 10⁶ cyanophage particles for some cyanophage strains (S-RSM2 and S-MM1; 5.3.4.3), and may be due to mismatching of the primers with the target cyanophage sequences. However, it is possible that there were high concentrations of cyanopodoviruses and cyanosiphoviruses at these stations, which would not have been detected by the cyanomyovirus-specific primers. As expected, virtually no product was obtained from station 10, at which the surface *Synechococcus* spp. concentration was very low (15°S; Fig. 6.3).

The samples which were concentrated for PCR were first filtered through 2 µm pore-size membranes to remove larger material yet retain *Synechococcus* spp. for future analysis (2.11.1). Hence, it may be possible that the primers amplified DNA from temperate cyanophages which had lysogenised their host. Indeed, Ogunseitan *et al.* (1992) used phage DNA probes to detect lysogenised bacteria as well as free phages. Direct PCR of bacterial colonies is possible (*e.g.* Gussow and Clackson, 1989); hence, it is not known whether the products are from free cyanophages or prophages.

However, removal of *Synechococcus* spp. by filtration appeared to have no effect on the amount of PCR product from coastal Trinidad seawater (Fig. 5.7), suggesting that the majority of product resulted from free cyanophages from this sample.

As mentioned earlier, a limitation of PCR is that it cannot distinguish infective from non-infective cyanophages. Since much research has shown rapid inactivation of marine viruses, especially near the surface where they are exposed to high UV irradiances (e.g. Suttle and Chen, 1992; Noble and Fuhrman, 1997), it may be that much of the PCR product is from inactivated cyanophages. However, these cyanophages are present nevertheless, and may have their infectivity restored by host-mediated repair mechanisms (Weinbauer *et al.*, 1997; Wilhelm *et al.*, 1997). Another limitation of PCR is the inability to detect bacteriocins (see 5.4.2).

6.3.1.2 Characterisation of marine cyanophage populations

RFLP analysis of the PCR products (Fig. 6.7) revealed that each station's products comprised a mixture of cyanophages containing all the possible restriction patterns. This illustrates the large diversity of each cyanophage population; however, changes in diversity between stations were not observed using RFLP analysis.

Time and resources only permitted the cloning and sequencing of PCR products from six stations, and many sequences were obtained only from two stations (6.2.4). More complete analysis of Atlantic cyanophage populations could have been made if every station's PCR products were sequenced, and if sequence data had been obtained from depth profiles. This leaves space for further analysis of the cruise samples.

The DNA and amino acid alignments (Fig. 6.8) reveal the high degree of conservation of the sequence in cyanophages when compared with T4, and suggests that the cyanophage-specific primers are amplifying only cyanophage sequences from the marine environment. In contrast, algal-virus-specific primers have been used to amplify sequences from a range of different marine algal viruses which infect

three genera of microalgae (Chen and Suttle, 1995a). The amino acid sequence of 18/1 (Fig. 6.8b) is vastly different due to a base deletion (see Fig. 6.8a). However, it is not certain whether this deletion belonged to the cyanophage, or may have been introduced during the PCR. Whilst this part of the T4 gp20 homologue appears to be very conserved, a high degree of variability exists at four residues (5, 16, 27 and 37).

The DNA alignment (Fig. 6.8a) shows that many of the sequences are identical (*e.g.* 2/1, 2/5, 2/6, 2/8, 2/14, 2/16, 2/23 and 2/39). It may be that these particular cyanophages were dominant in the marine sample. However, it has been shown that PCR of a mixture of two different target sequences does not always result in the same relative proportions as were initially present, but may give a bias towards a 1:1 final ratio (Suzuki and Giovannoni, 1996). However, Suzuki and Giovannoni (1996) suggested that this bias would likely be small in an environmental sample with highly diverse templates. Hence, the relative abundances of the different products may not necessarily accurately represent the initial relative abundances. Therefore, a multiplicity of a specific sequence may possibly be an artefact of the PCR, or of the cloning. Hence, it is not certain whether the cyanophage sequences obtained were representative of the cyanophages present in the initial sample.

Whilst sequence analysis of PCR products may not necessarily reveal the entire clonal variation within a cyanophage population, it does give considerably more information on cyanophage diversity than traditional microbiological techniques (see review by Fuhrman *et al.*, 1994). Using a combination of host range studies and TEM, Waterbury and Valois (1993) observed eight morphologically distinguishable cyanophages, belonging to different families, from a single seawater sample. In freshwater samples, using TEM, Demuth *et al.* (1993) observed up to eleven distinguishable phages, of indeterminate host, per sample. Yet, in a single sample from station 18 PCR identified at least twelve genetically different cyanophages (Fig. 6.8a), which were probably all cyanomyoviruses (according to the specificity of the primers; Table 5.1), and may well have looked morphologically virtually indistinguishable (see Frank and Moebus, 1987).

The dendrogram produced from the cyanophage DNA sequences (Fig. 6.9) illustrates the similarity between the different sequences, and gives the first ever reported information on genetic similarities of natural marine cyanophage populations. The dendrogram shows that some sequences amplified from the same sample are very similar to each other (*e.g.* 18/3, 18/15, 18/17, 18/18, 18/35/ and 18/37; and 2/1, 2/34 and 2/38). However, other sequences are very different from sequences in the same sample, and are more similar to those from different samples. In particular, sequence 2/9 is more similar to 18/37 than to all the other sequences from station 2. Indeed, Figure 6.8a reveals that 2/9 and 18/37 differ at just two bases. Yet 2/9 was from a sample between Port Stanley and Montevideo, whereas 18/37 was from a sample north of Cape Verde, off the west coast of Africa, about 4500 miles away in a distinct Atlantic gyre. Similarly, cyanophage strain S-RSM2 and 18/3 differ by just eight bases, yet originate from the Gulf of Aqaba, Red Sea, and north of Cape Verde, respectively, *i.e.* from different oceans.

Such conservation amongst widely separated cyanophages may point to the importance of a conserved structure and function of the T4 gp20 homologue amongst marine cyanophages (see review by Saier, 1996). However, Kellogg *et al.* (1995) suggest that an explanation of similar observations amongst vibriophages (see below) may be long-distance transport by cargo ships, which take on seawater as ballast (for references see Kellogg *et al.*, 1995). Such long-distance transport of cyanophages may more likely result naturally from ocean currents such as the Gulf Stream.

Similar observations have been made with different marine virus systems. Studying viruses which infected the photosynthetic flagellate, *Micromonas pusilla*, Cottrell and Suttle (1995) observed that genetic variation of viruses within a sample was as great as that of viruses from different oceans. In a similar, but less extensive study to this cyanophage study, Chen *et al.* (1996) investigated genetic diversity amongst the broad group of marine algal viruses (Phycodnaviridae). These viruses had been PCR-amplified directly from marine samples which had been concentrated by ultrafiltration. PCR products from a single offshore station in the Gulf of Mexico were cloned and analysed by RFLP. A selection of five clones was then sequenced,

and the genetic diversity observed was as great as previously observed for viruses of *M. pusilla* in different oceans (Cottrell and Suttle, 1995). However, unlike this study, Chen *et al.* (1996) did not compare sequence data from different locations. In a study of viruses which infected *Vibrio parahaemolyticus*, Kellogg *et al.* (1995) observed that populations of genetically similar vibriophages were widely distributed over large distances in the oceans, similar to the observations of cyanophages in this study.

Further analysis of the cyanophage populations obtained from the cruise could be performed using DGGE. This technique has the advantages of being more rapid than sequencing, and of giving a more complete and accurate view of the cyanophages present in the initial sample, since no cloning is involved. DGGE has revealed considerable information on the genetic diversity of environmental microbial populations (*e.g.* Muyzer *et al.*, 1993; Wawer and Muyzer, 1995; Teske *et al.*, 1996), and work has already begun in our laboratory on DGGE analysis of cyanophage populations from the cruise samples (Wilson, personal communication). Future work could involve DGGE comparisons of cyanophage strains present in a marine sample with corresponding *Synechococcus* spp. strains. *Synechococcus* spp. strains can be analysed using PCR of the RNA polymerase gene (*e.g.* Palenik, 1994), and the use of DGGE for analysis of cyanobacteria has recently been demonstrated using 16S rRNA amplification products (Nubel *et al.*, 1997). Analysis could be made of the distribution of specific cyanophage and *Synechococcus* spp. strains throughout depth profiles, and throughout the Atlantic transect, comparing them with other data obtained from the AMT-2 cruise, to gain deeper insight into the ecology of *Synechococcus* spp. and their cyanophages.

6.3.2 *Synechococcus* spp. and other cruise data

The contour plot of *Synechococcus* spp. abundance (Fig. 6.3) reveals a generally higher concentration nearer the surface, which decreases with depth, below 50-100 m. Comparison of the *Synechococcus* spp. count with the temperature profile (Fig.

6.5a) reveals that a thermocline between 40 and 80 m depth may be responsible for maintaining high *Synechococcus* spp. numbers in this surface mixed layer throughout much of the central part of the transect. Similar observations have been made previously (e.g. Iturriaga and Marra, 1988). In the higher latitudes the water was not yet stratified by a thermocline, and *Synechococcus* spp. were observed at greater depths. However, there appears to be no direct correlation between surface water temperature and *Synechococcus* spp. abundance, contrary to previous observations (e.g. Waterbury *et al.*, 1986).

Comparison of *Synechococcus* spp. abundance (Fig. 6.3) with the composite satellite data of chlorophyll *a* concentration (Fig. 6.4), shows that the three main peaks in *Synechococcus* spp. abundance occur in regions of greater productivity, manifested by greater chlorophyll *a* concentrations. These regions are: midway between Port Stanley and Montevideo (40-45°S); the west African region of upwelling (8-14°N); and west of the Bay of Biscay (40-46°N). High *Synechococcus* spp. concentrations ($8 \times 10^3 \text{ ml}^{-1}$) were also observed at the equatorial upwelling (ca. 0°N), with greater productivity visible here (Fig. 6.4). The expanse of oligotrophic open ocean in the South Atlantic, with very low productivity (Fig. 6.4), contained the lowest *Synechococcus* spp. abundance (8-32°S; Fig. 6.3). In contrast, Burkill *et al.* (1993) observed decreased *Synechococcus* spp. abundance at an upwelling region, and increased abundance in oligotrophic waters, in the Indian Ocean. However, Olson *et al.* (1990) observed a similar trend to this study, in a different transect of the Atlantic Ocean. The highest latitudes contained the greatest productivity (Fig. 6.4) yet low *Synechococcus* spp. concentrations (Fig. 6.3). This may be a result of productivity from other phytoplankton, such as diatoms, out-competing *Synechococcus* spp. (see review by Fogg, 1995). Indeed, high concentrations of eukaryotic phytoplankton, including a bloom of the coccolithophore *Emiliana huxleyi*, were observed SW of Plymouth (AMT-2 cruise report).

Comparison of the AMT-2 chlorophyll *a* data (Fig. 6.5b) with the composite satellite data (Fig. 6.4) reveals that the composite appears to be accurate for the period of the AMT-2 cruise. Figure 6.5b allows a more precise comparison of chlorophyll *a*

concentrations with *Synechococcus* spp. abundance (Fig. 6.3). The west African *Synechococcus* spp. peak (8-14°N) correlates very well with the corresponding peak in chlorophyll *a*, as do the troughs at 8-18°S. For much of the transect *Synechococcus* spp. abundance appears to correlate well with chlorophyll *a* concentration. However, low chlorophyll *a* concentrations were observed between 24 and 44°N (Fig. 6.5b), and yet this region contained high *Synechococcus* spp. counts (6.2). Hence, *Synechococcus* spp. may not contribute much of the total chlorophyll *a*. In support of this, Liu *et al.* (1995) observed that *Synechococcus* spp. contributed only a small fraction of total primary production in the North Pacific Ocean.

Comparison of the *Synechococcus* spp. counts (Fig. 6.3) with photosynthesis from the 0.2-2 µm fraction (Fig. 6.5c) shows a generally good correlation, with high *Synechococcus* spp. concentrations where photosynthesis is high. However, some regions contain high *Synechococcus* spp. concentrations but low photosynthesis (*e.g.* 6°S-4°N). These observations may point to the fact that the 0.2-2 µm fraction contains *Prochlorococcus* spp. as well as *Synechococcus* spp., and that a larger part of the photosynthesis may be from *Prochlorococcus* spp., as also observed by Liu *et al.* (1995). Total photosynthesis (Fig. 6.5d) shows a similar distribution to photosynthesis from the 0.2-2 µm fraction (Fig. 6.5c). In many regions the 0.2-2 µm fraction appears to contribute a large proportion, if not most, of the total photosynthesis (*e.g.* 20-30°N). Previous estimates have shown similar contributions of the *Prochlorococcus* spp./*Synechococcus* spp. size range to total production (Iturriaga and Marra, 1988; Liu *et al.*, 1995).

Comparison of *Synechococcus* spp. counts (Fig. 6.3) with bacterial counts (Fig. 6.5e) reveals a coincidence of high concentrations of both groups of organisms at the west African upwelling (8-14°N) and west of the Bay of Biscay (40-46°N). Like *Synechococcus* spp., bacterial counts are greatest in the surface mixed layer. These results support the idea that bacteria assimilate organic matter exuded from phytoplankton, in the microbial loop (Azam *et al.*, 1983), as has been commonly observed (*e.g.* Van Boekel *et al.*, 1992; Brussaard *et al.*, 1996b). These observations

also suggest that where cyanophage concentrations are high, there may also be high concentrations of bacteriophages.

Figure 6.5f shows that nitrate concentrations are very low in the surface 80 m (above the thermocline; Fig. 6.5a) for most of the transect, and at all depths in the open South Atlantic Ocean (10-36°S) illustrating the oligotrophic nature of the open oceans. The higher nitrate concentrations from *ca.* 0-14°N coincide with the equatorial and west African upwelling (Fig. 6.4), bringing up nutrients from deeper waters. Yet even here, nitrate concentrations are low in the surface mixed layer (above 50 m). Total photosynthesis rates are high in this surface region (Fig. 6.5d) suggesting that nitrate depletion is by phytoplankton activity in the euphotic zone, as expected (for review see Lalli and Parsons, 1993). Comparison of nitrate concentrations (Fig. 6.5f) with *Synechococcus* spp. counts (Fig. 6.3) illustrates that *Synechococcus* spp. are abundant in surface waters which are extremely oligotrophic, as has long been known (see review by Waterbury *et al.*, 1986). *Synechococcus* spp. concentrations increase where nitrate concentrations increase in the surface mixed layer (*ca.* 44°S), as expected (see review by Waterbury *et al.*, 1986).

Chapter 7:

Summary

The ecological importance of marine *Synechococcus* spp. has been known for some time (Waterbury *et al.*, 1986), yet information on the abundance and importance of cyanophages which infect these hosts has only appeared relatively recently (*e.g.* Suttle and Chan, 1993; Waterbury and Valois, 1993). Cyanophages are thought to play an important role in the mortality and clonal composition of marine *Synechococcus* spp. (Suttle and Chan, 1994; Waterbury and Valois, 1993), and have been shown to be widespread throughout the world's oceans (Suttle and Chan, 1993; 1994; Wilson, 1994). Concentrations of infective cyanophages have been shown to be commonly greater than 10^5 ml⁻¹ in surface waters (Suttle and Chan, 1993; 1994). However, relatively little research has been made into the molecular characterisation of cyanophages which infect marine *Synechococcus* spp. (Wilson *et al.*, 1993; Wilson, 1994).

The aims of this study were to continue research begun by William Wilson in this laboratory (Wilson, 1994) to develop molecular probes which would specifically detect cyanophages which infect marine *Synechococcus* spp., then to apply these probes to interrogate natural marine cyanophage populations. Molecular analysis was based on the PCR; hence, the probes were PCR primers. Another aim of this study was to develop a rapid and comprehensive technique for quantifying marine cyanophages, using cPCR.

For the subsequent development of cyanophage-specific PCR primers, sufficient controls were necessary. Attempts were made to isolate cyanophages, from different oceanographic provinces, which infected *Synechococcus* sp. strains WH7803 and WH8018 (3.2). Several cyanophages were isolated from coastal Bermuda and from Hydrostation S, in the Sargasso Sea, near Bermuda. These cyanophages were classified as belonging to the families Myoviridae and Podoviridae (Fig. 3.1). Correspondingly, most cyanophages which have been isolated on marine *Synechococcus* spp. belong to these two families, though the majority are myoviruses (*e.g.* Waterbury and Valois, 1993). DNA from cyanophages isolated in this study was probed by Southern analysis (Fig. 3.2) for cross-hybridisation with a 1 kb fragment from cyanophage strain S-PM2, which had been previously isolated (Wilson, 1994).

This fragment had been shown to cross-hybridise with DNA from all other marine cyanophages which had been screened (Wilson, 1994), and subsequently cross-hybridised with DNA from the cyanomyoviruses, but not the cyanopodoviruses, isolated in this study (Fig. 3.2). These results suggest that this region of DNA may be conserved amongst cyanomyoviruses.

Attempts were made to isolate bacteriophages which infect marine heterotrophic bacteria (3.4), but with no success; perhaps reflecting the low concentrations of specific bacteriophages in the marine environment. However, three marine bacteriophages, which belong to the same three families as all marine cyanophages, were kindly donated by Karlheinz Moebus. Southern analysis of these phages (Fig. 3.3) showed no cross-hybridisation with the cyanophage DNA fragment, suggesting that the region is conserved amongst cyanophages.

The 1 kb fragment from cyanophage strain S-PM2 had been partially sequenced (Wilson, 1994), and showed limited homology with T4 gene 20, which encodes a minor capsid protein (Arisaka *et al.*, 1988; Marusich and Mesyanzhinov, 1989). Homologous fragments from cyanophage strains S-WHM1 and S-BnM1 had been cloned into pUC19 (Wilson, 1994). Additional DNA fragments, containing the remaining parts of the T4 g20 homologue, were isolated from the three cyanophages (4.3), and the homologues were completely sequenced (Append. 1). These three DNA sequences were aligned with the homologous sequence from T4, and two PCR primers were designed from two regions which were conserved only in the three cyanophages, to make the primers cyanophage-specific (Fig. 4.9). The primers, termed CPS1 and CPS2, amplified a product of 165 bp following PCR.

Having designed putative cyanophage-specific primers, their specificity was assayed (5.3.2). PCR was attempted upon many marine cyanophages, including those isolated in Chapter 3, the majority of which were cyanomyoviruses. PCR was also attempted on various controls, including marine bacteriophages, viruses of marine eukaryotic phytoplankton, a freshwater cyanomyovirus and T4. The results (Fig. 5.2 and Table 5.1) showed that the primers detected only marine cyanophages which belonged to

the family Myoviridae, regardless of the geographical location of their origin. The cyanophage-specific primers also detected cyanophages regardless of their host range (Table 5.1), and therefore provide a more powerful and comprehensive tool for cyanophage detection than traditional methods, such as plaque assay, which are limited to the host strains available in culture. The large majority of cyanophages which have been isolated on marine *Synechococcus* spp. belong to the family Myoviridae (Suttle and Chan, 1993; Waterbury and Valois, 1993; Wilson, 1994). It is therefore probable that these primers would detect the majority of cyanophages in the oceans which infect marine *Synechococcus* spp..

To determine whether PCR would detect marine cyanophages in their natural concentrations, the sensitivity of the PCR was assayed (5.3.3). The primers were able to detect as few as 190 cyanophages, giving a visible PCR product (Fig. 5.6). The sensitivity was similar to that achieved with other virus systems using the same technique (*e.g.* Zammattéo *et al.*, 1995). This number of cyanophages would correspond to an *in situ* concentration of 10^3 PFU ml⁻¹ (5.3.3), well in the range of that commonly observed by Suttle and Chan (1993; 1994) in waters of the Gulf of Mexico. However, lower cyanophage concentrations could be concentrated by ultracentrifugation (5.3.3) or tangential flow filtration (6.2.4). Hence, the sensitivity of the assay should have been sufficient to detect most natural concentrations of marine cyanophages. Preliminary experiments showed that PCR products could be obtained from just 1 µl of unconcentrated seawater (Fig. 5.7), emphasising the sensitivity of the PCR. Previous PCR assays, of marine microalgal viruses, have required prior concentration of the viruses from seawater (Chen *et al.*, 1996). Even with a concentration step, PCR therefore provides a method of cyanophage detection which is far more rapid (*ca.* 5 h) than traditional infection techniques (*ca.* 5 days). The rapidity and simplicity of PCR also allows shipboard detection of cyanophages, so that real-time data can be obtained during a scientific cruise.

To enhance the usefulness of PCR for the interrogation of natural cyanophage populations, the development of a cPCR assay was attempted to quantify cyanophage populations (5.3.4), based on the strategy described by Zachar *et al.* (1993). An

internal competitor, based on cyanophage strain S-BnM1, was constructed (Fig. 5.10), and calibration curves were drawn for three cyanophages (Fig. 5.12), with a log-linear relationship over *ca.* three orders of magnitude of cyanophage numbers, similar to the range obtained by Lee *et al.* (1996). This demonstrates that rapid quantification of a known marine cyanophage is possible. Hence, cPCR would be very useful for enumerating cyanophages in culture studies. However, cPCR of the three different cyanophages resulted in three different calibration curves (Fig. 5.12). Hence, quantification of an unknown sample containing a mixture of cyanophages was not possible, with the degenerate primers used in this study. Therefore, natural populations of marine cyanophages could not yet be quantified by cPCR. For quantification, a more conserved sequence amongst marine cyanophages may be required.

Different methods were used to investigate cyanophage diversity, including RFLP analysis, bisbenzimid-PEG analysis and sequencing. Sequence analysis of PCR products, amplified by primers CPS1 and CPS2, proved the most successful for obtaining data on the diversity of marine cyanophages (5.3.5.3). Separation of the PCR products was attempted using bisbenzimid-PEG, (*e.g.* Wawer *et al.*, 1995), but with little success (5.3.5.2). However, DGGE analysis of cyanophages, using primers from this study (CPS4 and CPS5 with an added GC-clamp), has now been used to detect changes in cyanophage diversity through spacial and temporal scales using samples collected on the AMT-2 cruise (Wilson, this laboratory).

Whilst quantification of marine cyanophage populations was not yet possible by cPCR, rapid detection and molecular characterisation by sequence analysis of PCR products was possible. Hence, the cyanophage-specific primers were applied to marine samples which had been collected whilst on the AMT-2 cruise, from Port Stanley (Falkland Islands) to Plymouth (UK). *Synechococcus* spp. counts were made on the cruise, in anticipation of comparison with subsequent cyanophage counts by PCR, although the latter was not possible. However, the *Synechococcus* spp. counts (Fig. 6.3) suggested that there should be sufficient cyanophages throughout most of the transect, in the surface waters, for detection by PCR. Suttle and Chan (1994) had

shown that a threshold of *ca.* 10^3 *Synechococcus* spp. cells ml⁻¹ was necessary for efficient cyanophage replication, above which the cyanophage concentration increased considerably, to concentrations detectable by PCR (*ca.* 10^5 ml⁻¹).

The virus fraction was concentrated whilst on board the research ship, by tangential flow filtration, and PCR products were subsequently obtained from most of the surface samples (Fig. 6.6), demonstrating the presence of *Synechococcus* spp. cyanophages throughout the Atlantic Ocean. Products from some of the stations were sequenced, and a dendrogram was produced (Fig. 6.9). This is the first report of genetic information being obtained from natural marine cyanophage populations.

The results (Fig. 6.9) showed that these cyanophage populations were highly diverse, with at least twelve genetically different cyanomyoviruses in one sample (Fig. 6.8a). Whilst some sequences obtained from the same sample were clearly very similar to each other, others were very different. Cyanophages within a sample could be as diverse as those isolated from different oceans. Some cyanophage sequences were very similar to sequences obtained from stations thousands of miles away, in different hemispheres, or even in different oceans.

Future analysis of natural cyanophage populations could be made using sequence data or DGGE analysis based on cyanophage PCR products. Comparisons could be made between the diversity and distribution of cyanophages and marine *Synechococcus* spp. strains over horizontal, vertical or temporal scales, gaining a deeper insight into the ecological importance of marine cyanophages.

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Appendices

Appendix 1 DNA and amino acid sequence data containing homologues of T4 g20 in three cyanophages. The start and stop codons of the open reading frames are marked in the DNA sequences (a, c and e). Amino acid sequence data (b, d and f) were deduced from just the open reading frame. (a and b) Cyanophage strain S-PM2; (c and d) Cyanophage strain S-WHM1; (e and f) Cyanophage strain S-BnM1.

(a)

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1      GCAATCAACA CTCACAAACC AACAAACGCTC CTCTATTCAC TCCAAGTCTA GACTCTGTTA TGCCAGAATC
71     TCAGTTAGCA CTGAGAGATG AAGTGAGTCG GGTGAAGTTC TTAGAGAGTA TGTTCTCTAC TACGGATTCC
141    CAACTAATGT TTCTCAAATT GATGTTGATA TGACAGCAAC GATCAGATCG AGGAATTCAC AGTTGAGTTC
211    CAATACTCAT ACTGGAAGGT TGAATCTGGT ACTACACAAA ATGGAGTATC TGGAGTCAAG TCGGGAATCG
281    GTGATCAGCG CCTAGTAGAG AACTGATAAA TAGATCTATA GGTTAGGTCT ACTATATTAC TATGAGTCAA
351    CTCTTTGGTT TTTTAATAAA TGAAAAGAG GGACAGAAAG GTCAATCCCC TGTCCCTCCA AATGATGAAG
421    CATCCGTCAG TACTGTTGCT GCGCGATATT TTGGTACTTA TGTTGATACA TCTGGTGGAC AAAATTCAAG
491    AAACGAATAC GAACTCATTG GTAGATATCG TGATATGTCT CTCCACCCAG AAGTGGATAG TGCCATAGAC
561    GAAATCGTGA ATGAGTTTGT TGTTAATGAT GGCACGATA AACCCGTAGA AGTTGATCTT CAAAACCTAG
631    AAATTGGATC TGGTGTAAG AAAAAAATTC GCGATGAATT CAATAGAATT CTTCTGATGA TGAACTTTAA
701    TGTCACGCA CATGAAATTA TCAGAACTG GTATGTTGAT GGCAGATCAC ATTACCACAA AGTAATTGAC
771    TTAGATAATC CAAAGAAAGG TATCTTAGAA TTAAGATACA TCGATTCTCT AAAGATTAGA AAAGTTAGGC
841    AAAAATTAAG AGACGTAGAT CCCAATAGAA AGGAAATAGA AAAGGGAAGT GCTCTTCAAT ATGATTACGG
911    AGATTTTCATC GAATATTATA TTTACAATCC AAAAGGATTT GCAGGTAACA TCCCAATGGT TACTGGTTCG
981    ATGGATTGGT CAAACCAAGA AGGTATTAAG ATTGCGTCGG ATGCTATCGC GCAATCAACT TCTGGTTTGA
1051   TGGATCTAAA CAAAAGATG ACATTAAGCT TTCTCCATAA GGCAATCAAG TCTCTCAATC AACTAAGAAT
1121   GATCGAGGAC TCACTTGTTA TCTATCGTTT ATCTCGTGCT CCCGAGCGTA GAATTTTCTA TATTGATGTT
1191   GGTAATCTAC CAAAGGTAAA GGCAGAACAA TACTTGCGTG ATGTAATGTC ACGTTACAGA AACAAGCTTG
2061   TTTACGATGG TCAAACAGGA GAGATTCGTG ATGATAAAAA GCATATGTCT ATGCTGGAAG ATTTCTGGTT
2131   ACCTCGCCGT GAAGGTGGTA GAGGAACTGA GATCACTACA CTTCCAGGCG GTCAGAACCT TGGTGAATC
1401   AAGGATGTTG AGTATTTCAA AAAGAACTT TACAACCTAC TCAACCTACC ACCTTCCCGC CTTACGGATG
1471   ACAACAAAGC ATTTAATCTT GGTAAGTCCA CGGAGATCCT TCGAGATGAA CTCAAATTC AAGAGTTCAT
1541   TGGACGCCTT CGCAAACGTT TTGCACAACT TTTTCACGAT ATTCTCAAGA CGCAACTAAT CCTAAAGGGC
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1681   TCAATGAATT AAAAGAGCAA GAAATGCAAT TGCAGCGTGT CAATCTTGCT ACACAGATGG ATCCATTGTT
1751   TGGAAAGTAT TTTTCTACAG AATATATCCG TCGTAAGATT CTCATGCAAA CTGAAATGA ATTCAAAGAA
1821   ATTGATAAGC AAATGAAGTC TGACATTGAA TCTGGATTGG CAATCGATCC TATTCAAGTA AATATGCTTG
1891   ATGATATGGA AAAGCAAAT CAAGCATTG CTCCAGAACT TCAAGCAGCT CAGGATGATC TTGCTGCGGA
1961   AAGAGAAATT AAAAAATTGA ATTCCGCACC AAAACCCCT CCATCACAAC AATCAAATC TCAATCTAAT
2031   AAATAATGTT ATAGTTCAAT AATTAACAT GGATTCTGAA GTATTAGATA TTGTGAATTT TATTAGTGAT
2101   AAAAAGAGAG GAGATGCTCT AGA

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(b)

1 MSQFLGFLIN EKEGQKQSP VPPNDEASVS TVAGGYFGTY VDTSGGQNSR NEYELIRRYR DMSLHPEVDS
71 AIDEIVNEFV VNDGDDKPVE VDLQNLEIGS GVKKKIRDEF NRILRMMNFN VNAHEIIRNW YVDGRSHYHK
141 VIDLDNPKKG ILELRYIDSL KIRKVRQKLK DVDPNRKEIE KGTAQYDYG DFIEYYIYNP KGFAGNIPMV
211 TGSMDWSNQE GIKIASDAIA QSTSGMLDLN KKMTLSFLHK AIKSLNQLRM IEDSLVIYRL SRAPERRIFY
281 IDVGNLPPVK AEQYL RDVMS RYRNKLVDG QTGEIRDDKK HMSMLEDFWL PRREGGRGTE ITTLPGGQNL
351 GELKDVEYFK KKLNSLNLPSRLTDDNKA FNLGKSTEIL RDELKFTKFI GRLRKRFAQL FHDILKTQLI
421 LKGIITPEDW DMEEHIQYD FLFDNHFNEL KEQEMQLQRV NLATQMDPFV GKYFSTEYIR RKILMQTENE
491 FKEIDKQMS DIESGLAIDP IQVNMLDDME KQNQAFAPEL QAAQDDLAAE REIKKLNSAP KPPPSQQSKS
561 QSNK

(c)

1 CTGCAGACTC AGTATTGGAC TCCGATTATA GCAAACGACT GACCTGATAA ATAGACCAGG ACCAATAACT
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631 AACTTACACA GAAAGCAGCA GAGTATTTTC TTTACAACCC CAAGGGTTTG AAGAACTCTA CTAATCAGGG
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1681 TCTAAATAGA CTAATAATA CTACAGTGGG AACATATTAT GCCTAGTGAT ATTGCTAACA ATCGTCCACA
1751 GTGTATAGTG ACGAAAAA

(d)

1 MSQLFGFSLE RAKKVPKGPS FVQKDSMDGS QPIVGGGYYG YSVDFDGTVR NDYELISRYR EMVLQPECDs
71 AVDDIVNETI CGNFDDVPVE LELSNLKASD KIKKLMREEF DEVLRLLDfE NRAYEIFRRW YVDGRLfYHK
141 VIDPQNPRGG LAELRYIDPR KIRKvTEYEQ KRPEQLRGVD INTQLTQKAA EYFLYNPKGL KNSTNQGMKI
211 TTDSVCYCHS GIQDLNKNMT LSHLHKAika VNQLRMIEDS LVIYRLSRAP ERRIFYIDVG NLPKNKAEQY
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(e)

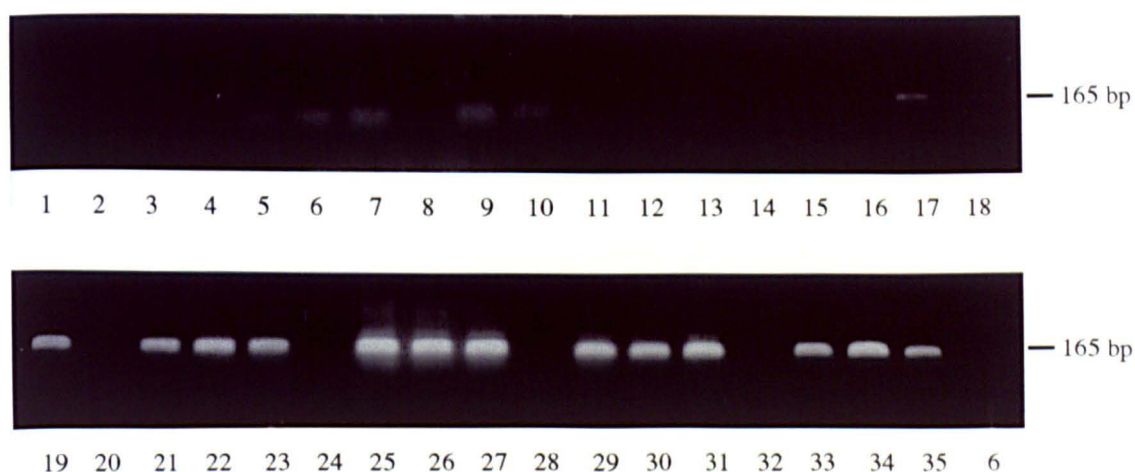
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211 GATGGACAAG TATCCATAGC TGCTGGTGGG CACTATGGGT ACTTTGTCTGA TCTAGAAGGT GGTGGGAAAA
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841 CTTTCATACC TCCATAAAGC AATCAAAGCA CTCAATCAAC TTCGCATGAT TGAGGACTCT CTGGTCATCT
911 ATCGTTTATC ACGCGCACCA GAACGTAGAA TTTTCTACAT TGATGTTGGC AATCTTCCAA AGGTAAAGC
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1681 TTAGGTGATG TTCCTATGGA TCCGAGGCA GATGGTTCCG CGACTGAACC ACCAGAAGGA CAAGGACTTA
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(f)

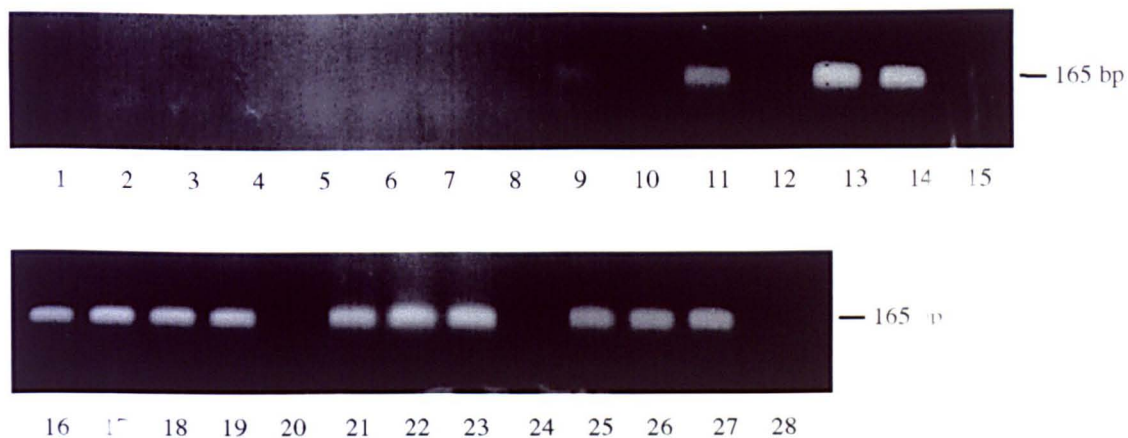
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141 YHKMIDFDDP SKGITEIRYV DPQKIRLVKE VQKNADQTRT DLNNKYDYG VLEYIYNPK GLKQNPMSA
211 TAPTGGGIKI AKDAITFVQS GLLDANKGMV LSYLHKAIKA LNQLRMIEDS LVIYRLSRAP ERRIFYIDVG
281 NLPKVKAQEQY LREVMGRYRN KLVYDANTGE IKDDKKHMSM LEDFWLPRRE GGRGTEISTL PGGQNLGELT
351 DVEYFKKKLY RALNVPIISM EAEGGFNLGR SSEILRDEIK FTKFVGRLRK KFSEIFIDML KTQLILKGIT
421 SVEEWEDMKE YIQFDIFYDN HFSELKESEL LQERINLASA ADPYVGKYFS VEYVRSKILH QTDTEMDMD
491 DQIEKEKDAG IIPSEEEMM AMQGGEGGA LGDVPMDPEA DGSATEPPEG QGLI
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Appendix 2 Optimisation of PCR conditions for the putative cyanophage-specific primers CPS1 and CPS2.

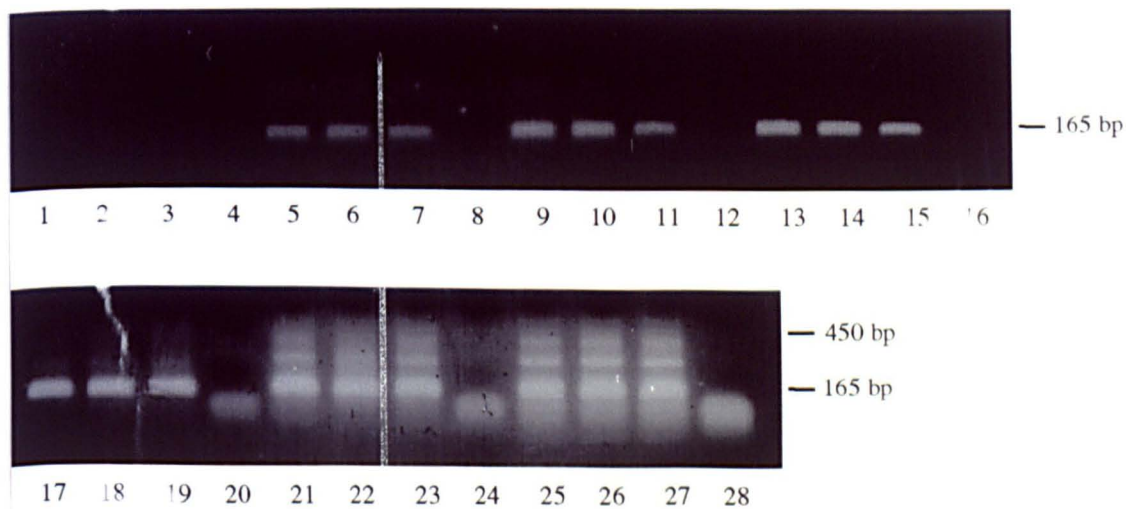
The conditions which are optimised are: (a) cycle number; (b) MgCl_2 concentration; and (c) concentration of each primer. For each of the subsequent tests, the previous optimised condition was used. The initial amount of target DNA was kept at 100 ng per reaction, and the annealing temperature was kept at 55°C . The resulting reaction mixtures, some containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h. For each condition triplicates were made, and a negative control with no target DNA added.



(a) **Cycle number.** Tracks 4, 8, 12, 16, 20, 24, 28, 32 and 36 are negative controls. Tracks contain reaction mixtures from the following numbers of cycles: (1-4) 5; (5-8) 10; (9-12) 15; (13-16) 20; (17-20) 25; (21-24) 30; (25-28) 35; (29-32) 40; (33-36) 45.

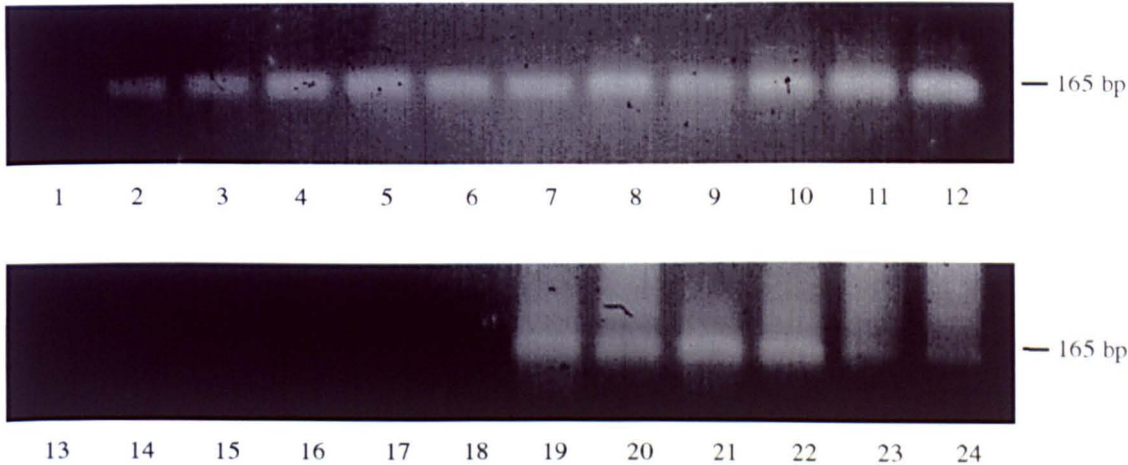


(b) **MgCl₂ concentration, mM.** Tracks 4, 8, 12, 15, 20, 24 and 28 are negative controls. Tracks contain reaction mixtures from the following MgCl₂ concentrations, in mM: (1-4) 0.25; (5-8) 0.6; (9-12) 1.25; (13-16) 2.5; (17-20) 5; (21-24) 10; (25-28) 25.

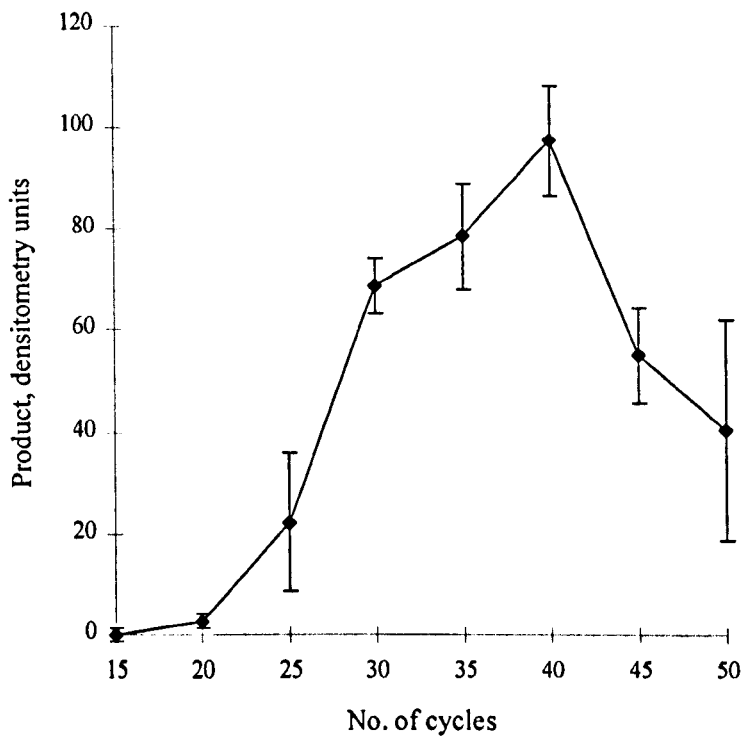


(c) **Primer concentration, nM.** Tracks 4, 8, 12, 16, 20, 24 and 28 are negative controls. Tracks contain reaction mixtures from the following primer concentrations, in nM: (1-4) 2; (5-8) 5; (9-12) 10; (13-16) 20; (17-20) 40; (21-24) 100; (25-28) 200.

Appendix 3 Optimisation of cycle number for cPCR. PCR was performed using 100 pg of DNA from cyanophage strain S-BnM1, with the cyanophage-specific primers, CPS1 and CPS2. The resulting reaction mixtures, some containing the 165 bp products, were run on a 1.2% (w/v) agarose gel at 60 mA for 1 h (a). Products were quantified by scanning laser densitometry and plotted against cycle number (b).



(a) Agarose gel electrophoresis of PCR products. Tracks contain products from the following number of cycles: (1-3) 25; (4-6) 30; (7-9) 35; (10-12) 40; (13-15) 15; (16-18) 20; (19-21) 45; (22-24) 50.



(b) Graph of product concentration with increasing number of cycles. The mean of triplicate samples is plotted, with standard deviation bars.

Appendix 4 Statistical comparison of cyanophage calibration curves. Linear regressions of the cPCR calibration curves for cyanophage strains S-MM5 and S-BM3 are compared with each other to determine whether the gradients are significantly different. Calculations are taken from Zar (1996). The null hypothesis (H_0) is that the gradients are equal. Terms with the subscript 1 are for cyanophage strain S-MM5; terms with the subscript 2 are for cyanophage strain S-BM3.

$$b = \frac{\sum xy}{\sum x^2} \quad \text{residual SS (sum of squares)} = \frac{\sum y^2 - (\sum xy)^2}{\sum x^2}$$

residual DF (degrees of freedom) = $n - 2$

$$\frac{(s^2_{y \cdot x})_p = \text{residual SS}_1 + \text{residual SS}_2}{\text{residual DF}_1 + \text{residual DF}_2}$$

$$s_{b_1 - b_2} = \sqrt{\left[\frac{(s^2_{y \cdot x})_p}{\Sigma x_1} + \frac{(s^2_{y \cdot x})_p}{\Sigma x_2^2} \right]}$$

$$t = \frac{b_1 - b_2}{s_{b_1 - b_2}} \quad v = \text{residual DF}_1 + \text{residual DF}_2$$

S-MM5:

$$\Sigma x^2 = 243.31 \quad \Sigma xy = 15.67 \quad \Sigma y^2 = 4.288 \quad n = 8$$

S-BM3:

$$\Sigma x^2 = 240.64 \quad \Sigma xy = -1.159 \quad \Sigma y^2 = 2.901 \quad n = 8$$

Hence, $t = 1.061$

According to the statistical table, at the 95% confidence interval, where $v = 12$, $t_{0.05(2), 12} = 2.179$.

Hence, since the value of t is not greater than 2.179, H_0 is not rejected. Therefore, the regressions are not significantly different at the 95% confidence interval.

Appendix 5 AMT-2 cruise samples. Date (Julian day) and location of each station on the AMT-2 cruise (1996) from the Falkland Islands to Plymouth (UK), together with the samples which were concentrated by tangential flow filtration for cyanophage detection.

Station	Julian Day	Latitude	Longitude	Samples (depths, m)
1	114	47° 38.0' S	55° 38.0' W	7; 20; 30; 40; 50; 70
2	115	43° 25.6' S	54° 44.2' W	7
3	116	39° 24.3' S	53° 19.5' W	7; 20; 40; 60; 80; 100
4	120	36° 03.1' S	49° 48.7' W	7
5	121	33° 23.0' S	46° 37.5' W	7; 20; 40; 60; 90; 120
6	122	30° 18.0' S	43° 25.2' W	7
7	123	27° 37.1' S	40° 42.6' W	7; 30; 50; 70; 90
8	124	24° 21.2' S	37° 51.9' W	7
9	125	19° 54.3' S	35° 31.3' W	7; 50; 80; 100
10	126	15° 10.3' S	33° 18.4' W	7
11	127	11° 14.7' S	31° 27.7' W	7; 30; 70; 130
12	128	07° 28.8' S	29° 45.3' W	7
13	129	03° 55.0' S	28° 12.6' W	7; 30; 60
14	130	00° 11.7' S	26° 24.1' W	7; 20; 40; 70; 80
15	131	03° 24.1' N	24° 47.7' W	7
16	132	07° 34.6' N	22° 56.6' W	7; 30; 40; 50; 70
17	133	11° 44.4' N	21° 03.1' W	7
18	135	19° 54.3' N	21° 19.2' W	7; 30; 50; 70; 100
19	136	26° 32.3' N	21° 47.7' W	7
20	137	30° 55.0' N	21° 16.3' W	7; 50; 60; 80; 100; 120
21	138	35° 39.9' N	20° 25.1' W	7
22	139	39° 53.9' N	19° 59.8' W	7; 20; 40; 50; 80; 120
23	140	44° 07.7' N	20° 01.1' W	7
24	141	46° 29.7' N	14° 03.8' W	7
25	142	48° 45.0' N	08° 05.8' W	7